INOSITOL #89

Monograph on **Inositol** #89 7/18/73

M O N O G R A P H

O N

I N O S I T O L

TR-72-1552-32

Submitted Under:
Contract No. FDA 72-104

July 18, 1973

INFORMATICS INC.
6000 Executive Boulevard
Rockville, Maryland 20852

INOSITOL

Summary

Inositol was discovered in 1850 by J. Scherer, who isolated it from animal muscle tissue. Although the compound isolated by Scherer was myo-inositol, over the years the name inositol has come to be used as a generic term for the entire family of isomeric hexahydroxy-cyclohexanes. However, myo-inositol is the only compound discussed in this monograph, and when the term inositol is used hereafter, it will be understood to signify myo-inositol.

Inositol is widely spread throughout nature in both plant and animal tissues and, therefore, is a part of the human daily diet to the extent of approximately 1 g per day. Williams estimated that this amount is the probable human requirement (574). Numerous studies have shown that inositol functions as a vitamin for various species of experimental animals, and the compound is now generally classified as a member of the vitamin-B complex. Although inositol has not been shown to be essential for man, that is, no definite symptoms of inositol deficiency have yet been produced in man, the extent of the occurrence of inositol in the human body and its association with the phospholipids of liver, brain, spinal cord and other tissues indicate that it probably functions as a vitamin in the human body.

The widespread occurrence of inositol, its vitamin activity, and its use as a food additive have resulted in considerable research into its biochemistry and toxicology.

The results of tests on the absorption and metabolism of inositol are, as of yet, inconclusive. Wiebelhaus et al. (567) found that 24-28 hours were required for the complete absorption of doses of 250 mg inositol by fasted, 200 g rats. Doses of 800 mg inositol administered to 250 g rats caused no appreciable changes in the level of this compound in the blood, liver or testes, but did produce a significant increase in the heart content (567). Anderson (11) reported that inositol is absorbed very slowly from dog intestine in situ. Sonne and Sobotka (498) found daily ingestion of 1.5 g of inositol to produce, in human subjects, a moderate rise of the plasma inositol level.

Studies by Stetten and Stetten (506) provided evidence to indicate a minimum of 7% conversion of injected inositol (2 g) to glucose in rats. Using white rats, Wiebelhaus et al. (567) found no conversion of inositol to liver glycogen, but did observe an antiketogenic effect and reduced urinary nitrogen excretion, indicating that inositol is utilized as a carbohydrate. They also found that urinary excretion accounts for less than 1% of the administered inositol when given to 200 g rats in doses of 260-355 mg (567). On the basis of studies using nephrectomized rats, Howard and Anderson (258) concluded that the kidney is the only organ of importance in inositol catabolism. Moscatelli and Larner (373) and Coots (98) reported that inositol is vigorously metabolized to CO_2 by the rat, up to 40% of the administered dose being excreted into the respiratory

CO_2. Kotaki et al. (286) found no significant differences in the urinary excretion of inositol among groups of young albino rats treated orally with 0-50 mg of inositol per day.

Upon finding that as much as 77% of administered inositol was recovered from the excreta of dogs and that it effected no rise in the RQ, Anderson (11) and Dubin (128) concluded that inositol is not utilized to any extent by the dog. Following subcutaneous injection of inositol solution in phlorizinized dogs, Greenwald and Weiss (203) observed a slight but unmistakable increase in the urinary glucose: nitrogen ratio and concluded that, in the dog, inositol is slowly and incompletely converted to glucose.

On the basis of experiments in which ^{14}C-labelled inositol was administered to a normal human subject and was incubated with slices of rabbit kidney cortex, Freinkel et al. (174) suggested that the metabolism of inositol occurs via the glucuronic acid pathway and pentose cycle. Following the ingestion, by a human subject, of 30 g of inositol one day and 10 g the next morning, Anderson and Bosworth (12) found that 9% of the ingested inositol was eliminated in the urine, within 24 hours, and that the feces contained none.

Several physiological effects of inositol have been reported. The lipotropic action of inositol in rats has been investigated and confirmed by numerous workers (186, 187, 188, 49, 48). Abels, Kupel, Pack and Rhoads (3) in clinical studies on human patients having gastrointestinal cancers and livers heavily infiltrated with fat, found that administration of inositol reduced liver fat to a nearly normal level in most cases in less than 24 hours. Preoperative oral administration of inositol to humans has been reported to result in a marked reduction of total liver lipids (3, 2, 16). Best et al. (48) reported that inositol (30 mg/day) was not effective in preventing the occurrence of the hemorrhagic kidneys which developed in young rats on diets low in choline and methionine and Handler (214) found that although inositol exerted lipotropic activity in the liver, it actually appeared to increase, slightly, the incidence and severity of renal hemorrhages in rats due to choline deficiency. Upon treating old hens with 0.5 g inositol per day for periods up to 68 days, Herrmann (244) was able to discern a definite decrease in the cholesterol content of the blood and tissues of the birds. Martin et al. (349) reported that the peroral administration of inositol (20 mg/kg) markedly increases the peristaltic action of the stomach and small intestine of dogs without creating a spastic condition. Bly et al. (57) and Anderson (11) reported that inositol administration produced diarrhea in dogs, and Anderson and Bosworth (12) reported a similar effect in man. Vorhaus et al. (544), however, reported that the administration of 1-2 g of inositol per day to 20 human patients produced in no case any change in the normal bowel habits.

The synergism of the lipotropic action of inositol and choline was first pointed out by Forbes (167) and later confirmed by a number of other workers (52, 54, 213, 335, 451). Martin (348) reported that the addition of p-aminobenzoic acid to a highly purified ratio produced a syndrome, characterized by poor growth and changes in fur in the Rockland strain black rat, which could be prevented by the

administration of inositol, and conversely, the addition of inositol to the diet in the absence of p-aminobenzoic acid caused a similar syndrome which could be prevented by p-aminobenzoic acid.

Toxicological studies on inositol have been restricted, so far, to rats and man. No information is available on the acute toxicity of inositol.

Two studies on the influence of inositol on the reproduction of rats have been reported. Ershoff (141) found that inositol administered as 1% of the diet produced no adverse effects on the growth and reproduction of Long-Evans rats. Sure (515), however, observed that inositol (30 mg/day, oral) has a pronounced injurious effect on lactation in albino rats.

In studying the carcinogenicity of inositol, Laszlo and Leuchtenberger (310) and Hesselbach and Burk (246) found that intravenous injections of inositol inhibit tumor growth in mice.

By giving 20-day-old male Wistar rats daily oral doses of 0, 0.01, 0.1, 0.2, and 1.0 g inositol/kg Natsume (383) determined that doses of 0.01-0.1 g/kg/day exerted a favorable effect on growth rate, while 1 g/kg/day caused slight growth retardation. In a comparative study in which 3-month-old rats were given 0, 0.005, 0.05, 0.5 and 5 g inositol/kg/day for 1 month, no inhibitory effect on growth was seen among the animals given large dosages of inositol (383). A two-month feeding study by Kotaki et al. (286) indicates that young albino rats maintained on a basal diet exhibited repressed growth and an increased P/N ratio of hepatic lipids when given high levels of inositol (50 mg/day, oral).

Studies of inositol administration to man have revealed only slight effects. Maurer and Seckfort (353) reported that intravenous injection of 4 g inositol to healthy subjects produced no effects other than a reduction of basal metabolism (15-25%). Anderson and Borworth (12) reported that a human subject given, per os, 15 g of inositol on one day and 30 g/day for 3 days thereafter exhibited only mild diarrhea and increased urinary excretion of creatinine. Vorhaus et al. (544) administered 1-2 g of inositol daily to patients for periods of over two months without the production of any adverse effects. Shay (544) reported similar findings. Minor increases in total and free cholesterol and total phospholipids and an apparent decrease in neutral fat in the plasma of 13 patients suffering from high tone deafness were noted by McKibben and Brewer (359) following treatment with 3 g of inositol (oral) per day for periods of 1-12 months. Following i.v. injection of 2 g inositol/day to 12 patients for 16 days, Staubach and Seckfort (504) observed no adverse effects.

INOSITOL

Chemical Information

I. Nomenclature

A. Common Names

1. Inositol
2. meso-Inositol
3. myo-Inositol
4. i-Inositol
5. Hexahydroxycyclohexane
6. Cyclohexanehexol
7. Cyclohexitol
8. Meat sugar
9. Inosite
10. Mesoinosite
11. Phaseomannite
12. Dambose
13. Nucite
14. Bios I
15. Rat antispectacled eye factor
16. Mouse anti-alopecia factor
17. Phaseomannitol
18. Inosin
19. Muscle sugar

B. Chemical Names

1,2,3,5/4,6-Cyclohexanehexol

C. Trade Names

None

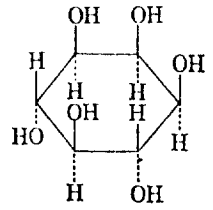
D. Chemical Abstracts Registry Number

000087-89-8

II. Empirical Formula

$C_6H_{12}O_6$

III. Structural Formula



IV. Molecular Weight

180.16

V. Specifications

Food Chemicals Codex	
Assay	Not less than 97.0% of $C_6H_{12}O_6$ after drying
Melting range	Between 224 degrees and 227 degrees
Limits of impurities	
Arsenic (as As)	Not more than 3 ppm
Calcium	Passes test
Chloride	Not more than 50 ppm
Heavy metals (as Pb)	Not more than 20 ppm
Lead	Not more than 10 ppm
Loss on drying	Not more than 0.5%
Residue on ignition	Not more than 0.1%
Sulfate	Not more than 60 ppm

VI. Description

A. General Characteristics

Inositol occurs as fine, white crystals or as a white crystalline powder. It is odorless and has a sweet taste.

B. Physical Properties

Inositol has nine possible stereoisomers, seven of which are inactive or meso. Two optically active forms, the racemic form, and several cis, trans-somers occur naturally. The prevalent natural form is cis-1,2,3,5-trans-4,6-cyclohexanehexol, which has the following properties: d 1.752; mp 225-227 degrees; optically inactive; solubility in water at 25 degrees = 14 g/100 ml soln, at 60 degrees = 28 g/100 ml soln; slightly soluble in alcohol; practically insoluble in ether and other common organic solvents; aqueous solutions are neutral to litmus.

C. Stability

Inositol is stable in air.

VII. Analytical Methods

The method of Platt and Glock for the determination of inositol utilizes the unique behavior of inositol toward periodic acid oxidation as the basis of the determination. In this procedure the test-material is extracted with water, and the aqueous extract further extracted with 70 per cent acetone and then with ether. The fractions insoluble in 70 per cent acetone and soluble in ether are discarded, and glucose removed from the extracted aqueous solution by yeast fermentation. The

acidic and basic substances which may be present in the aqueous solution are then removed by selective adsorption on appropriate ion-exchange resins. The free inositol remaining in the purified water solution after the above treatment is finally oxidized quantitatively with periodic acid, the excess of periodic acid being estimated iodometrically. Water-soluble combined inositol is determined after acid-hydrolysis of the aqueous extract (420).

VIII. Occurrence

A. Plants

Inositol is found widely distributed in many different types of plants. It is most frequently encountered in combination with phosphoric acid, although free inositol and monomethyl and dimethyl ethers of inositol have also been isolated from some plant species. The most common form for the occurrence of inositol in plants is as a hexaphosphoric acid ester called phytic acid, which is found to be heavily concentrated in seeds and cereal grains, accounting for as much as 86% of the total phosphorus present.

B. Animals

Inositol was first discovered by isolation from animal muscle tissue and has subsequently been found in many of the vital organs of the human and animal body. The human tissues richest in inositol are brain, stomach, kidney, spleen and liver, in decreasing order of inositol concentration. The richest animal-food sources of inositol are brain and heart. As with plants, much of the inositol in animals and man appears in combined form.

C. Synthetics

None

D. Natural Inorganic Sources

None

Biological Data

I. Acute Toxicity

Man

In studying the effect of inositol on the basal metabolism of man, Maurer and Seckfort injected, intravenously, 21 patients with 2 g inositol dissolved in 20 ml of 0.9% NaCl, 10 patients with 4 g inositol in the same solvent, and 14 patients with the solvent alone. In all cases the inositol injected was endured without complications. Those patients receiving 4 g of inositol showed a distinct reduction (15-25%) in their basal metabolic rates. The effect of 2 g inositol was questionable (353).

II. Short-term Studies

Rats

To study the effects of inositol ingestion, Natsume gave an unspecified number of 20-day-old male Wistar rats (average weight 50 g) myoinositol in oral doses of 0, 0.5, 5, 10, and 50 mg/animal/day for a period of 45 days. The administration of 0.5-5 mg inositol/rat/day exerted a favorable effect on the growth rate of the rats, but the level of 50 mg/animal/day produced slight growth retardation, evident 10-15 days after onset of administration (see Fig. 1). In 4 out of the 24 animals given the highest dosage of inositol there appeared staining of the hair due to excessive fatty secretion, loss of appetite and subsequent general hyposthenia. However, these symptoms showed no reproducibility and could not be attributed to the large dose of inositol. Analysis of urine collected from the rats revealed no significant difference in the amount of inositol excreted by animals receiving various dosages. Changes in the pattern of lipids in the liver were investigated and it was found that the larger the dosage of dietary inositol, the larger the P/N ratio of hepatic lipids, although there were no significant changes in the relative amounts of the total lipids and cholesterol in the liver. In a comparative experiment, an unspecified number of 3-month-old rats were given, orally, 0, 0.005, 0.05, 0.5, or 5 g inositol/kg/day for 1 month. With these older rats, no inhibiting effect on growth was seen among the animals given large dosages of inositol (383).

In a two-month feeding study by Kotaki et al., groups of 3 to 8 young albino rats were maintained on a basal ration with supplements of inositol and choline as depicted in Table 1 on the following page. At the end of the feeding period the rats were sacrificed and tested for serum and hepatic total lipids and the hepatic lipids were tested for N, P, and cholesterol content. As shown in Figure 1, on the following page, high levels of inositol were found to repress growth. Serum and liver total cholesterol were not significantly affected. However, the P/N ratio of hepatic total lipids increased with increased inositol administration. Further tests showed that these effects were diminished by elevating the protein content of the ration, or by increasing the amount of choline administration (286).

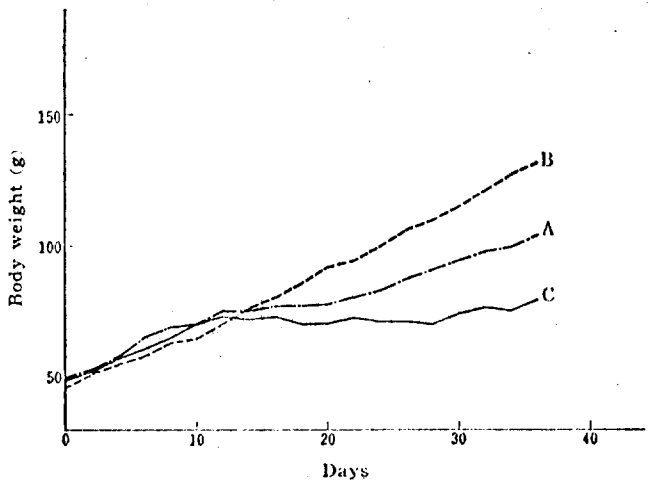


FIG. 1 *Growth Repressing Effect of Myoinositol when Administered in a Large Excess Amount*

A, no addition; B, 0.5 mg/day; C, 50 mg/day of myoinositol.

TABLE 1

Administration of Myoinositol and Choline

Experimental group	Addition	
	Myoinositol	Choline
	mg/day	*mg/day*
	Exp. 1	
A	—	1.25
B	0.5	1.25
C	5	1.25
D	10	1.25
E[a]	50	1.25
	Exp. 2	
F	—	1.25
G	0.5	1.25
H	5	10
I	10	20
J[a]	50	100

[a] Administered as suspension.

Man

In an effort to discover whether the ingestion of inositol produced any change or disturbance in the metabolism of man, Anderson and Borworth administered inositol to a human subject per os for 4 days at a level of 15 g on the first day and 30 g/day thereafter. The urine was collected and analyzed for total nitrogen, uric acid, creatinine, ammonia nitrogen, total phosphorus, inorganic phosphorus, organic phosphorus, and inositol. The feces were also collected and were analyzed for nitrogen and phosphorus. Except for mild diarrhea during the first few days, the only effect produced by this regimen was increased excretion of creatinine for several days following the ingestion period (12).

Vorhaus et al. administered from 1 to 2 g of inositol daily to patients for periods of over two months without the production of any adverse effects (544).

In a discussion of the work of Vorhaus et al., Shay stated that he administered 1.2 g of inositol per day to humans for a considerable period without any ill effects (544).

In an effort to determine the effect of inositol ingestion on the concentration of plasma lipid inositol and several other plasma lipids, McKibben and Brewer treated a group of 13 patients suffering from high tone deafness with 3 g of inositol (oral) per day for periods of 1-12 months. No change resulted in the fasting plasma inositol phosphatide concentration; there were minor increases in total and free cholesterol and total phospholipids, and there was an apparent decrease in neutral fat. None of the patients reported any untoward symptoms or reactions from the inositol (359).

Staubach and Seckfort examined the effects of inositol on man by injecting, intravenously, 2 g doses of inositol into 12 patients in a single dose and in another 12 patients daily for 10 days (all patients were either healthy or mildly ill). Blood samples were taken at regular intervals throughout the test period and were tested for both qualitative and quantitative aspects. Neither qualitative structural nor quantitative concentration changes of the peripheral blood cells were observed (504).

III. Long-term Studies

None

IV. Special Studies

Reproduction

To study the effects of inositol (1% of diet) on reproduction, Ershoff employed 42 female Long-Evans rats, which were selected at 23 days of age and an average weight of 41.8 g. They were divided into groups of 6 and fed ad libitum the 7 diets shown in Table 1 on the following page. They were weighed weekly, and vaginal smears were taken

daily from the 48th day. After the 60th day the rats were bred to males of proven fertility. No adverse effects on growth or reproduction were noted in the animals fed purified rations supplemented with inositol. Lactation was inadequate with both the control and the experimental rations, although some young were weaned on each of the diets (141).

TABLE I.
Composition of Experimental Diets.

Dietary component	A	B	C	D	E	F	G
Casein*	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Salt mixture†	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Sucrose	65.5	64.5	63.5	64.5	63.5	65.5	55.5
p-aminobenzoic acid		1.0	2.0		1.0		
Inositol				1.0	1.0		
Folic acid						0.0005	
Yeast‡							10.0

To each kg of diets A-F, were added 80 mg thiamine hydrochloride, 20 mg riboflavin, 20 mg pyridoxine hydrochloride, 100 mg calcium pantothenate, 100 mg nicotinic acid, 5 mg 2-methyl naphthaquinone and 1.2 g choline chloride. To each kg of diet G were added 5 mg 2-methyl-naphthaquinone and 1.2 g choline chloride. In addition each rat on diets A-G received the following supplement 3 times weekly: 500 mg cottonseed oil (Wesson), 1.0 mg alpha-tocopherol, and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 50 U.S.P. units of vitamin D.‖

* Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

† Sure's Salt Mixture No. 1.5

‡ Brewers' type yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

‖ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

The effect of inositol on reproduction and lactation and the interaction of inositol and p-aminobenzoic acid was studied by Sure in the albino rat. Groups of female rats were maintained on various rations as shown in Table 2. In the first 3 groups, inositol was administered in daily doses of 10-15 mg per animal until mating and in 30 mg doses during the reproduction and lactation periods, while p-aminobenzoic acid was fed in 3 mg daily doses per animal during the first 4 weeks followed by 7.5 mg doses until breeding, and 15 mg doses during pregnancy and lactation. The other groups received the test compounds in their rations at a level of 0.2%. As evidenced by the data in Table 2, inositol administration resulted in a very marked harmful effect on lactation. The concurrent administration of p-aminobenzoic acid, however, produced a pronounced protective action against inositol injury.

TABLE 2

The role of p-aminobenzoic acid and inositol in lactation.

RATION NO.	GROUP NO.	SOURCE OF PROTEIN	SOURCE OF CARBOHYDRATES	SPECIAL ADDITIONS	P-AMINOBENZOIC ACID OR INOSITOL ADDITIONS	NO. OF FEMALES MATED	NO. OF LITTERS	TOTAL YOUNG BORN	YOUNG GIVEN TO REAR	YOUNG REARED	PER CENT YOUNG REARED
13	1	Casein, 10% Fibrin, 10%	Dextrin	Dried grass, 2%	Control	5	6	64	36	6	16.6
13		"	Dextrin	"	inos.[1,2]	5	6	56	35	0	0.0
13		"	Dextrin	"	p.a.b.-inos.[1,2]	5	8	65	41	16	39.0
12	2	Casein, 10% Fibrin, 10%	Dextrin	Liver concentrate, 1%	Control	5	10	127	60	20	33.3
12		"	Dextrin	"	inos.[1]	5	13	96	63	10	15.9
12		"	Dextrin	"	p.a.b.-inos.[1]	5	9	106	54	33	61.0
14	3	Casein, 10% Fibrin, 10%	Dextrin	Dried liver, 2%	Control	5	9	92	40	23	57.5
14		"	Dextrin	"	inos.[1]	5	9	88	53	5	9.4
14		"	Dextrin	"	p.a.b.-inos.[1]	5	11	109	60	51	85.0
8	4	Fibrin 25%	Dextrin	Yeast nucleic acid, 0.2%	Control	5	10	97	59	18	30.5
9		Fibrin, 25%	Dextrin	"	inos., 0.2%	5	6	44	23	5	21.7
10		Fibrin, 25%	Dextrin	"	p.a.b., 0.2%	5	6	67	36	18	50.0
11		Fibrin, 25%	Dextrin	"	p.a.b., 0.2% inos., 0.2%	5	6	50	16	5	31.2
7	5	Casein, 22.5%	Cerelose		Control	10	13	117	77	17	22.1
2	5	Fibrin, 25%	Cerelose		Control	15	32	340	216	87	40.3
3		Fibrin, 25%	Cerelose	Polished rice, 30%		5	11	96	66	44	66.6
4		Fibrin, 25%	Cerelose	"	inos., 0.2%	5	12	112	67	21	31.3
5		Fibrin, 25%	Cerelose	"	p.a.b., 0.2%	5	11	112	63	62	98.4
6		Fibrin, 25%	Cerelose	"	inos., 0.2% p.a.b., 0.2%	5	10	108	60	53	88.3
1		Fibrin, 25%	Dextrin		Control	15	21	195	119	33	27.7

[1] Administered separately from the ration in doses outlined in the text.

[2] p.a.b. = p-aminobenzoic acid; inos. = inositol.

As shown in Table 3, Laszlo and Leuchtenberger found that intravenous injections of inositol inhibit tumor growth in mice. Subcutaneous and oral administration of inositol were found to be ineffective (310).

TABLE 3

EFFECT ON TUMOR GROWTH OF FOUR INTRAVENOUS INJECTIONS OF INOSITOL IN VARYING DOSES GIVEN OVER A PERIOD OF 48 HOURS*

Group No.	No. of animals in each group	Dose of Inositol γ	Mean terminal tumor weight mg	Standard error
453	11	0 (control: saline)	470	25.6
452	18	38	436	22.8
451	14	50	350	33.6
450	10	75	270	34.1
449	7	100	246	41.1
448	5	150	215	26.4
447	5	250	222	9.8
446	5	1000	142	12.8

*Female Rockland mice transplanted with Sarcoma 180; start of the experiment 8 days after transplantation; mice kept on polished rice diet for the experimental period of 48 hours.

In similar tests, Hesselbach and Burk confirmed the tumor-inhibiting activity of inositol (246).

I. Breakdown

The procedure and results of tests by Cheldelin et al. on the losses of inositol by foods due to cooking are presented in Table 1. Inositol losses among meats were quite variable, but were moderate in most cases. In general, smaller losses were observed among samples which had been subjected to relatively mild treatment such as steaming. Inositol losses in the cooking of vegetables were often much greater than those encountered for meats, with especially high losses among the legumes. The authors stated that in view of the stability of inositol toward heat, this is surprising especially since the severity of heat treatment for the vegetables tested was much less than that for most of the meats. They suggested, therefore, that the losses observed may be due to "binding" of the inositol in various tissue combinations rather than to thermal destruction (91).

Food	Manner of cooking	Time of cooking	Water or fat added	Weight before cooking	Weight after cooking	Total content		Loss
						Before	After	
Meats		*Minutes*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*%*
1.* Beef round	Fried in open pan	15	1	40	36	18	16	11
2. Beef liver	Fried in open pan	10–15	1	40	35	22	15	32
3. Beef liver	Fried, covered pan	10	16	94	75			
4. Beef heart	Steamed	30	93	190	210	420	420	0
5. Beef heart	Steamed	30	50	100	150	290	330	0
6. Pork loin	Fried in open pan	10–15	0	40	29	18	13	28
7. Pork loin	Fried in open pan	10–15	0	45	28	20	13	35
8. Pork loin	Fried in open pan	10–15	0	68	43	25	20	20
9. Bacon	Fried in open pan	5	0	41	17	18	7.5	58
10. Bacon	Fried in open pan	3–5	0	22	13	14	12	14
11. Ham	Fried in open pan	15	0	54	30	17	18	0
12. Ham	Fried in open pan	15	0	59	37	34	27	21
13. Veal chop	Fried in open pan	10–15	0	42	30	15	16	0
14. Veal chop	Steamed	30	84	170	180	54	32	39
15. Lamb leg	Roasted, 300° F.	120	0	690	550	400	310	24
16. Mutton shoulder	Steamed	30	66	133	199	68	77	0
17. Chicken leg	Fried in open pan	10–15	0	41	35			
18. Chicken leg	Fried in open pan	10–15	5	69	46	26	22	15
19. Chicken breast	Fried in open pan	10–15	0	31	29	10	10	0
20. Chicken breast	Fried in open pan	10–15	0	31	29	21	14	33
21. Salmon	Fried in open pan	20	3	111	97	19	19	0
22. Halibut	Fried in open pan	7–10	2	126	104	24	18	25
23. Halibut	Steamed	30	0	5	5	.7	.7	0
Vegetables								
24. Beets	Boiled, covered kettle	40	266	133	124			
25. Cooking water					140			
26. Beets	Steamed	40	190	380	370	80	43	46
27. Beet tops	Steamed	10	20	40	60	8.3	3.9	54
28. Cabbage	Steamed	30	25	100	125	95	62	35
29. Carrots	Steamed	30	25	50	75	24	24	0
30. Cauliflower	Steamed	20	120	120	240	96	93	3.1
31. Cauliflower	Steamed	30	50	100	150	110	60	46
32. Onions	Fried in open pan	20	29	208	53			
33. Potatoes	Boiled, covered kettle	30	200	190	200			
34. Cooking water					190			
35. Potatoes	Boiled, covered kettle	20	360	180	210			
36. Cooking water					210			
37. Lima beans	Steamed	60	10	5	15	8.4	3.3	61
38. Okra	Steamed	20	15	30	45	16	16	0
39. Rice	Steamed	25	60	30	90	7.9	3.1	61
40. Sauerkraut	Steamed	30	0	5	5	1.5	1.2	20
41. Spinach	Steamed	10	50	100	150	27	20	26
42. Split peas	Steamed	40	100	50	150	160	7.8	95
43. Sweet potatoes	Baked	45–60	0	2200	1700			
44. Turnips	Boiled, covered kettle	40	114	114	118			
45. Cooking water					90			
46. Turnips	Steamed	20	22	22	44	10	11	0
Fruit								
47. Apples	Boiled, open kettle	20–30	1360	3400	2300			
Eggs and milk								
48. Eggs	Scrambled, open pan	10–15	8	150	139			
49. Eggs	Scrambled, open pan	10–15	1	50	46			
50. Eggs	Baked	30	0	100	100	19	20	0
51. Eggs	Scrambled, covered pan	5	0	100	94			
52. Milk	Heated, glass dish	30	0	100	100			
53. Milk	Heated, open metal pan	15	0	100	100			

II. Absorption - Distribution

Figure 1 is a graphic representation of the results obtained by Wiebelhaus et al. following administration, by gastric intubation, of inositol in doses of about 250 mg to fasted, 200 g rats. A period of 24-28 hours was required for complete absorption. It was determined in the study that no conversion of administered inositol to a bound form had occurred in the intestine. In further studies the administration of 800 mg inositol to 250 g rats caused no appreciable changes in the level of this compound in the blood, liver or testes, but did produce a significant change in the heart content. The hearts of fasted rats were found to contain 95-97 mg% of inositol while those of inositol-fed rats contained 123-152 mg% (567).

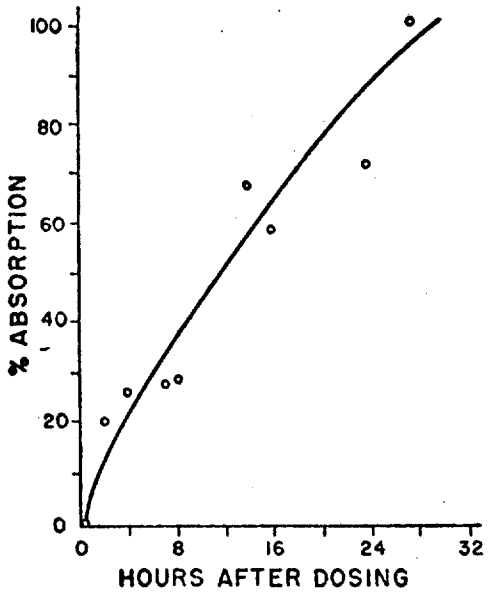


FIG. 1. Rate of inositol absorption in fasted rats.

Anderson found inositol to be absorbed very slowly from dog intestine in situ (11).

Using a nephelometric micro bioassay with Saccharomyces carlsbergensis, Sonne and Sobotka found the range of plasma inositol in one normal human subject and four with amyotrophic lateral sclerosis to be 0.37-0.76 mg/100 ml for individual fasting samples. Pooled plasma from miscellaneous patients ranged from 0.54 to 1.87 mg/100 ml. Daily ingestion of 1.5 g of inositol was found to produce, in most cases, a moderate rise of the plasma inositol level (498).

III. Metabolism and Excretion

Stetten and Stetten reported that the intraperitoneal injection

of deuterium-labeled inositol (2 g in 16 ml water given in 4 doses at intervals of 2.5 hours) into a phlorizinized rat resulted in the excretion of urinary glucose containing sufficient deuterium to indicate a minimum of 7% conversion of the injected inositol to glucose (506).

Wiebelhaus et al. found no increase in the liver glycogen of white rats after the administration, by stomach tube, of inositol. However, inositol, given by stomach tube or intraperitoneally, functions antiketogenically in the white rat and at the same time reduces the amount of total nitrogen excreted in the urine, which suggests that the material given is utilized as a carbohydrate in the fasted animal without a prior conversion to glycogen as such. With 200 g white rats, the urinary excretion of inositol was found to vary from 3 to 5 mg/day/rat and was not significantly greater for rats which had received 260-335 mg of inositol per os than for fasted control rats. It was concluded that urinary excretion accounts for less than 1% of the administered inositol (567).

Howard and Anderson found that inositol-2-^{14}C is not degraded to respiratory $^{14}CO_2$ by nephrectomized rats. On the basis of this finding and the results of earlier tracer experiments, they concluded that the kidney is the only organ of importance in inositol catabolism. In further experiments they found that rat kidney slices could convert at least 4.7 micromoles of inositol-2-^{14}C to $^{14}CO_2$/g tissue/hour at inositol concentrations (in the incubation medium) of 12 mM or greater, which suggests that a rats kidneys can catabolize all of the inositol ingested in an average daily diet (258).

Following intraperitoneal injection of ^{14}C-labelled inositol into normal adult male white rats, Coots found that the inositol was vigorously metabolized to CO_2 by the rat, 20-40% of the administered dose being excreted into the respiratory CO_2 within 8 hours. Inositol was also found to be incorporated intact into complex tissue components, such as phosphous lipids, and was found to be converted into glucose. Incorporation of ^{14}C into glycogen was found to be quite slow, periods greater than 3 hours being required to attain appreciable specific activity (98).

Three pathways of inositol metabolism in the intact rat were quantitatively investigated by Moscatelli and Larner by the intraperitoneal injection of ^{14}C-labelled inositol. A small conversion to glucose, amounting to 0.12-3.16%, was shown by liver glycogen labeling. Comparable amounts were incorporated into organ lipids, the liver and kidneys showing the highest levels (0.32 and 0.20% of the dose, respectively). Vigorous oxidative dissimilation of ^{14}C-inositol to $^{14}CO_2$ was found to occur in the intact rat, with 10.1-25.7% of the administered doses being expired in this form (373).

Table 1 shows the results of a study by Kotaki et al. in which they found no significant differences in the urinary excretion of inositol among groups of young albino rats treated with various levels of oral inositol (286).

TABLE I

Urinary Excretion of Myoinositol

Amount of myoinositol administered	Amount of myoinositol in 24 hour urine
mg/day	*mg*
—	0.182
0.5	0.155
5	0.256
10	0.140
50	0.176

[a] Average value of three animals.

Anderson observed that when administered to dogs perorally at the level of 2 g/kg of body weight, inositol does not cause a rise in the respiratory quotient. As much as 77% of the administered dose was recovered from the excreta, only a small portion of which was eliminated via the kidneys. It was concluded that inositol is not utilized to any extent by the dog (11).

The findings of Anderson (11) in the dog were corroborated by a later study by Dubin (128).

Following subcutaneous injection of inositol solutions in phlorizinized dogs, Greenwald and Weiss observed a slight but unmistakable increase in the urinary glucose: nitrogen ratio. They concluded that, in the dog, inositol is slowly and incompletely converted to glucose (203).

On the basis of experiments in which ^{14}C-labelled inositol was incubated with slices of rabbit kidney cortex and was administered to a normal human subject, Freinkel et al. suggested that the metabolism of inositol occurs via the glucuronic acid pathway and pentose cycle (174).

Following the ingestion, by a human subject, of 30 g of inositol one day and 10 g the next morning, Anderson and Bosworth found that a small quantity of the ingested inositol (9%) was eliminated in the urine and that the feces contained none. Excretion of unchanged inositol was complete within 24 hours after ingestion. The fate of the remaining 91% of the ingested material was not determined (12).

IV. Effects on Enzymes and Other Biochemical Parameters

The lipotropic effect of inositol was discovered in 1941 by Gavin and McHenry. In their original paper and in subsequent publications, they reported a type of fatty liver in rats which they believed to have been caused by the presence of excessive amounts of biotin in the experimental diet. Inositol was found to be particularly effective in treating the fatty livers produced by this diet, and to possess marked lipotropic action. However, inositol was found to have no effect on thiamine-induced fatty livers in rats except in the presence of other B vitamins (186, 187, 188).

Best, Lucas, Patterson and Ridout, working with rats having

fatty livers induced by dietary means, confirmed the lipotropic action of inositol (49, 48).

Abels, Kupel, Pack and Rhoads, in clinical studies on human patients having gastrointestinal cancers and livers heavily infiltrated with fat, found that administration of inositol reduced liver fat to a nearly normal level in most cases in less than 24 hours (3). Pre-operative oral administration of inositol to humans has been reported to result in a marked reduction of total liver lipids (3, 2, 16).

Hemorrhage in the kidneys is one of the corollary effects to fatty infiltration of the liver, and is particularly characteristic of the syndrome caused by choline deficiency. Best et al. reported that inositol (30 mg/day) was not effective in preventing the occurrence of the hemorrhagic kidneys which developed in young rats on diets low in choline and methionine (48). Handler found that although inositol exerted lipotropic activity in the liver, it actually appeared to increase, slightly, the incidence and severity of renal hemorrhages in rats due to choline deficiency (214).

Upon treating (orally) old hens with 0.5 g of inositol per day for periods up to 68 days, Herrmann was able to discern a definite decrease in the cholesterol content of the blood and tissues of the birds. The author concluded that inositol is a decholesterizing agent and effects some mobilization of cholesterol and cholesterol esters from the tissues (244).

Martin et al. reported that the peroral administration of inositol (20 mg/kg) markedly increases the peristaltic action of the stomach and small intestine of dogs without creating a spastic condition (349).

In enterostomized dogs maintained exclusively on a peeled whole wheat bread diet, Bly et al. showed that a severe deficiency developed in 2-3 months, and that this condition was accompanied by a diminution of gastrointestinal motility and 40-60% decreases in the rates of carbohydrate and protein digestion and absorption. A supplement of pantothenic acid was shown to restore the function to normal, but inositol acted more as a cathartic (57).

Anderson noted diarrhea in a dog following administration of 2 g of inositol per kg of body weight. He suggested that this phenomenon is caused by the slow absorption of the alcohol (11).

Anderson and Bosworth reported that when inositol is ingested by man at the rate of about 0.5 g/kg of body weight/day it produces some diarrhea at first or frequent soft stools. After a few days the stools, although more frequent than usual, are nearly of normal consistency (12).

Vorhaus et al. reported that the administration of 1-2 g of inositol per day to 20 human patients produced in no case any change in the normal bowel habits (544).

V. Drug Interaction

The synergistic action of inositol and choline has been fairly well established. Forbes was the first to point out that simultaneous administration of inositol and choline to rats, on certain diets, results in a greater reduction in the concentration of liver lipids than if either substance is given alone (167). This synergistic effect of inositol has since been confirmed by a number of workers (52, 54, 213, 335).

Martin reported that the addition of p-aminobenzoic acid to a highly purified ration produced a syndrome, characterized by poor growth and changes in fur, which could be prevented by the administration of inositol. The addition of inositol to the diet in the absence of p-aminobenzoic acid caused a similar syndrome, which could be prevented by p-aminobenzoic acid (348).

VI. Consumer Exposure Information

In 1942, Williams calculated that a good, mixed diet, furnishing 2500 calories per day, supplies approximately 987 mg of inositol per day. Assuming this amount to be that needed on a 2500 calorie mixed diet, he estimated the probable human requirement of inositol as approximately 1 g or more per day. The diet from which he calculated this value is shown in Table 1 (574).

TABLE 1.—*Vitamin B Content of Foods*

Constituent	Per Cent of the Calories	Thiamine, Mg.	Nicotinic Acid, Mg.	Riboflavin, Mg.	Pantothenic Acid, Mg.	Biotin, Mg.	Inositol, Mg.	Pyridoxine, Mg.	Folic Acid, Mg. Unit
Whole milk.....	20	0.3	0.4	1.4	2.2	0.06	100	0.20	0.100
Whole wheat.....	30	1.0	11.5	0.34	2.4	0.01	350	0.44	0.366
Lean meats									
Beef.....	9	0.07	10.0	0.4	1.0	0.01	21	0.11	0.043
Pork.....	9	1.5	7.0	0.2	0.7	0.01	8	0.11	0.025
Liver.....	2	0.07	5.0	1.0	2.4	0.13	23	0.02	0.057
Potatoes.....	10	0.2	3.6	...	1.0	0.002	87	0.66	0.390
Butter.....	5	*	*	*	*	*	*	*	*
Eggs.....	6	0.2	*	0.04	0.2	0.002	1	*	*
Fresh peas.....	4	0.15	2.0	0.14	0.7	0.014	162	0.05	0.124
Fresh carrots....	4	0.1	0.6	0.15	0.6	0.012	235	0.16	0.285
Orange juice.....	2	*	*	*	*	*	*	*	*
Total.....	100	3.6	40.1	3.67	11.2	0.25	987	1.77	1.39

* The amount of these foods is small enough so that they contribute a negligible amount of the B vitamin in question to the diet.

The inositol content of various biological materials is presented in Table 2 (482).

TABLE 2

THE INOSITOL CONTENT OF VARIOUS BIOLOGICAL MATERIALS

	Inositol content, γ/g. of fresh tissue	Reference
Vegetable Products		
Cereals		
Wheat germ	6,900	4
Whole wheat	1,700	4
	1,900[a]	5
Bread, whole wheat	644	6
	1,030	4
Flour, white	830	4
Flour, whole wheat	1,105	6
Oats	3,160[a]	5
	1,000	7
Barley	3,920[a]	5
Corn	500	7
Fruits		
Oranges	2,100	4
Orange juice	1,040–1,700 (per ml.)	8
Grapefruit	1,500	4
Grapefruit juice	880–1,120 (per ml.)	8
Cantaloupe	1,200	4
Tomatoes	460	4
Apples	240	4
Vegetables		
Peas, English, green	1,620	4
Cabbage	950	4
Potatoes, sweet	660	4
Potatoes, white	290	4
Lettuce	550	4
Carrots	480	4
Spinach	270	4
Miscellaneous		
Peanuts, roasted	1,800	4
Molasses	1,500	4
	3,200[b]	9
Tea leaves, dry	10,000	8
Yeast, *Torulopsis utilis*	2,700	10
Yeast, brewer's	500	7
Animal Products		
Meats		
Beef, muscle	115	4
	2,640	7
Beef, heart	2,600	4
	16,000	7
Beef, brain	2,000	4
	6,000	7
Beef, liver	3,400	7
	510	4
Pork, loin	360–450	4
Veal, chop	320–350	4
Fowl and Fish		
Chicken, breast	480	4
Oyster	440	4
Halibut	170	4
Salmon	170	4
Egg	220	7
Milk Products		
Whole milk, cow	500	7
	180 (per ml.)	4
Whole milk, human	330 (per ml.)	4
Cheese	250	4

[a] Determined as phytin phosphorus. These inositol weights were calculated from Courtois and Perez values for phytin phosphorus, assuming a formula $Ca_5Mg(C_6H_{12}O_{24}P_6 \cdot 3H_2O)_2$ for phytin.

[b] This included 1340 γ of free inositol and 1860 γ of bound (phytin) inositol.

The following tables were compiled from data submitted by user firms. Food consumption values for each food category were derived from the Market Research Corporation of America (MRCA) data on frequency of eating and from the USDA data on mean portion size of foods in each food category. The food consumption values thus derived were coupled with the usage level data obtained in the surveys to calculate the daily intake of each substance.

Table 3 reports the usage of Inositol in infant formulas and baby foods. Table 11 reports the annual poundage data and table 13 reports the possible daily intake per food category and total dietary based on food consumption by total sample.

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/02/72 PAGE 2

TABLE 3 -- USAGE LEVELS REPORTED FOR NAS APPENDIX A SUBSTANCES (GROUP II) USED IN INFANT FORMULA PRODUCTS & BABY FOODS(B)

NAS SURVEY NO.	SUBSTANCE NAME	FDA CATEGORY NO.	NAME	# FIRMS REPORTING	*** USUAL USE *** WTD MEAN, %	*** MAXIMUM USE *** WTD MEAN, %
0099	INOSITOL	83	FORMULAS(B)	*	.00346	.00372

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/03/72 PAGE 5

TABLE 11, PART A -- ANNUAL POUNDAGE DATA FOR NAS APPENDIX A SUBSTANCES (GROUPS I & II)

SUBSTANCE NAME (SURVEY NO.)	# REPORTS TO NAS 1960/1970	POUNDAGE REPORTED TO NAS (MATCHING REPORTS FOR BOTH YEARS) 1960	1970	TOTAL 1970 POUNDAGE REPORTED TO NAS	# REPORTS TO FEMA	POUNDAGE REPORTED TO FEMA-- 1970 ONLY	TOTAL 1970 POUNDAGE NAS + FEMA
INOSITOL NAS 0099	*/ *	400	7,133	7,133			7,133

COMPREHENSIVE GRAS SURVEY -- NAS/NRC 1972 10/02/72 PAGE 25

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY. BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE -- SEE EXPLANATORY NOTES IN PRECEDING SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO.	NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH S
INOSITOL NAS 0099	83	FORMULAS(B)	*	0-5 MO.	11.615220	21.279000	12.466040
				6-11 MO.	2.366640	11.276140	2.544480
				12-23 MO.	.761200	.214520	.818400
INOSITOL NAS 0099	ALL CATEGORIES		*	0-5 MO.	11.615220	21.279000	12.488040
				6-11 MO.	2.366640	11.276140	2.544480
				12-23 MO.	.761200	.214520	.818400

Bibliography

1. Abedi-Djourabtschi, M., and F. Hartmann. 1954. The effect of streptomycin, Aureomycin and meso-inositol on the lipids of rat liver. Hoppe-Seyler's Zeitschr. Physiol. Chem. 298(3/5):193-198.

* 2. Abels, J. C., I. M. Ariel, H. T. Murphy, G. T. Pack, and C. P. Rhoads. 1944. Metabolic studies in patients with cancer of the gastrointestinal tract. IX. Effects of dietary constituents upon the chemical composition of the liver, especially in patients with gastrointestinal cancer. Ann. Internal Med. 20:580-589.

* 3. Abels, J. C., C. W. Kupel, G. T. Pack, and C. P. Rhoads. 1943. Metabolic studies in patients with cancer of gastro-intestinal tract. XV. Lipotropic properties of inositol. Proc. Soc. Exp. Biol. and Med. 54(1):157-158.

4. Abrams, R. E. 1952. Vitamins. Bull. Am. Coll. Apothecaries 13(5):7-11.

5. Ackermann, D., and P. H. List. 1959. The occurrence of taurobetaine, taurine, and inositol in the giant siliceous sponge. Z. Physiol. Chem. 317:78-81.

6. Agranoff, B. W., and M. R. S. Fox. 1959. Antagonism of choline and inositol. Nature 183:1259-1260.

7. Ahluwalia, B. S., et al. 1966. Free carbohydrates in seminal plasma and sperm cells of semen of fowl. J. Reprod. Fertil. 12:359-61.

8. Alfano, A., and G. Amati. 1949. Action of vitamin D on peripheral blood and on marrow in normal subjects. Boll. Soc. Ital. Biol. Sper. 25:1017-19.

9. Alstroem, I. 1954. Effect of inositol on the rate of phosphorus metabolism in the liver of rats. Acta Med. Scand. 148:433-8.

10. Anderson, L., and R. H. Coots. 1958. Metabolism of 2-14C-myo-inositol in the rat. Biochim. et Biophys. Acta 28(3):666-667.

* 11. Anderson, R. J. 1916. The utilization of inosite in the dog. J. Biol. Chem. 25:391-397.

* 12. Anderson, R. J., and A. W. Bosworth. 1916. The utilization of inosite in the animal organism. The effect of inosite upon the metabolism of man. J. Biol. Chem. 25(3):399-407.

13. Angelico, R., and M. Quintilani. 1952. Pantothemic acid as growth factor in albino rats in relation to varying vitamin integration of the diet. Rend. Ist. Super. Sanita (Rome) 15:235-60.

14. Angyal, S. J. 1957. The inositols. Quart. Revs. (London) 11:212-36.

15. Aoe, H., and I. Masuda. 1967. Water-soluble vitamin requirement of carp. II. Requirements for p-aminobenzoic acid and inositol. Nippon Suisan Gakkaishi 33(7):674-80.

* 16. Ariel, I. M., J. C. Abels, H. T. Murphy, G. T. Pack, and C. P. Rhoads. 1944. Metabolic studies in patients with cancer of the gastrointestinal tract. VIII. The chemical composition of the liver, especially in patients with gastrointestinal cancer. Ann. Internal Med. 20:570-579.

17. Arima, K. 1950. Bioassay of vitamins. Kagaku no Ryoiki (J. Japan. Chem.) 4:30-44, 88-98, 143-52.

18. Arrigo, L., and T. Montini. 1952. Increase of cholesterol in the tissues: antagonists and the role of some organs in the genesis. Athena (Rome) 18:17-24.

19. Asada, K., and Z. Kasai. 1962. Formation of myo-inositol and phytin in ripening rice grains. Plant Cell Physiol. (Tokyo) 3(4):397-406.

20. Ash, A. S. F., and T. M. Reynolds. 1955. Water-soluble constituents of fruit. V. Sugars and polyols of the apricot fruit. Australian J. Chem. 8:444-50.

21. Ata, S., I. Ito, and N. Iso. 1951a. The effect of inositol on the growth and metabolism of the tubercle bacillus. Nagoya J. Med. Sci. 14:77-8.

22. Ata, S., I. Ito, and N. Iso. 1951b. Dihydrostreptomycin resistance of Mycobacterium tuberculosis. Nagoya J. Med. Sci. 14:135-7.

23. Atkin, L. 1949. Yeast growth factors. Wallerstein Lab. Communic. 12(37):141-151.

24. Atkin, L., P. P. Gray, W. Moses, and M. Feinstein. 1949. Growth and fermentation factors for different brewery yeasts. Wallerstein Lab. Communic. 12(37):153-170.

25. Baier, W. E., and T. C. Manchester. 1949. Inositol and folic acid in citrus fruit. Calif. Citrograph 34:361-4.

26. Baker, H., O. Frank, I. Pasher, B. Black, S. H. Hutner, and H. Sobotka. 1960. Growth requirements of 94 strains of thermophilic bacilli. Can. J. Microbiol. 6:557-63.

27. Balaguer-Vintro, I. 1954. Atherosclerosis induced by cholesterol and the mechanisms of its inhibition. Rev. Espanola Fisiol. 10(1):1-8.

28. Balatre, P., and M. Traisnel. 1955. Colorimetric determination of inositol. Bull. Soc. Pharm. Lille 1:28-9.

29. Balduni, M., S. Mainoli, and O. Piccinelli. 1952. The lipotropic action of inositol and some other substances in experimental hepatitis. Arch. Sci. Biol. (Italy) 36:581-601.

30. Balduini, M., G. Ghia, and C. Ghia. 1953. Liver-protecting action of liver extracts and of vitamins of the B group. Arch. Sci. Med. 95:85-108.

31. Banerjee, B., and C. H. Chakrabarti. 1966. Influence of niacin and inositol on faecal excretion of cholesterol and bile acid of albino rats kept on high cholesterol diet. J. Nutr. Diet 3(3):72-74.

32. Barnabei, O., and C. Gieri. 1949. Action of some vitamins on the oxidation of ascorbic acid. Ann. Univ. Ferrara 7(Pt. 1):123-42.

33. Barnett, J. E. G., A. Ralph, and K. A. Munday. 1970. Structural requirements for active intestinal transport. Nature of the carrier-sugar bonding at C-2 and the ring oxygen of the sugar. Biochem. J. 118(5):843-50.

34. Bather, B., et al. 1964. Inositol and the protection of cells. II. The release of nucleic acids from normal and malignant cells. Canad. J. Biochem. 42:167-77.

35. Bather, R., et al. 1965. Evidence for the role of bound water and partial desiccation in carcinogenesis. Nature (London) 207:30-2.

36. Battaglia, F. C., G. Meschia, and D. H. Barron. 1960. Determination of free myoinositol in biological fluids and tissues. Biochim. et Biophys. Acta 44:354-5.

37. Baumgarten, F. 1955. Paper chromatography to investigate blood and urinary carbohydrates in healthy and diabetic persons. Deut. Z. Verdauungs-U. Stoffwechselkrankh. 15:198-210.

38. Beadle, G. W. 1945. Genetics and metabolism in Neurospora. Physiol. Rev. 26:643-63.

39. Beatty, C. H., and E. S. West. 1951. The effect of substances related to the tricarboxylic acid cycle upon ketosis. J. Biol. Chem. 190:603-10.

40. Beckmann, R. 1953. Physiologic and clinical significance of meso-inositol. Medizinische, pp. 998; 1003-1005.

41. Belli, L., and S. Montorsi. 1953a. Inositol does not explain the effect of the antihistamines. Boll. Soc. Ital. Biol. Sper. 29:131-4.

42. Belli, L., C. Ghiringelli, and S. Montorsi. 1953b. The capillary-protective action of inositol as shown by various tests. Boll. Soc. Ital. Biol. Sper. 29:134-7.

43. Bendandi, A., and G. C. Manfredi. 1954. Variations of hepatic sulfhydryl groups during lipotropic therapy. Arch. Intern. Pharmacodynamie 98:152-60.

44. Benitez, H. H., M. R. Murray, and E. Chargaff. 1954. Inhibition of colchicine effect on mitosis. Ann. N.Y. Acad. Sci. 58:1288-1302.

45. Bergamini, A., A. Bendandi, G. Chierego, and G. Maggi. 1955. The enzymic picture of hepatic steatosis after a hyperlipide-hypoprotein diet: variations after lipotropic treatment. Boll. Soc. Ital. Biol. Sper. 31:521-3.

46. Bergamini, A., A. Bendandi, G. Maggi, and G. Chierego. 1955. Action of lipotropic substances on the enzymic picture of hepatic tissue with steatosis produced by carbon tetrachloride. Boll. Soc. Ital. Biol. Sper. 31: 800-801.

47. Bessey, O. A., H. J. Lowe, and L. L. Salomon. 1953. Water-soluble vitamins. Ann. Rev. Biochem. 22:545-628.

* 48. Best, C. H., C. C. Lucas, J. M. Patterson, and J. H. Ridout. 1946a. Lipotropic properties of inositol. Science 103:12-13.

* 49. Best, C. H., C. C. Lucas, J. M. Patterson, and J. H. Ridout. 1946b. Influence of biotin upon relative lipotropic effects of choline and inositol. Biochem. J. 40:368-373.

50. Best, C. H., C. C. Lucas, J. M. Patterson, and J. H. Ridout. 1951. The rates of lipotropic action of choline and inositol under special dietary conditions. Biochem. J. 48(4):452-458.

51. Best, C. H., J. H. Ridout, J. M. Patterson, and C. C. Lucas. 1951. A statistical evaluation of the lipotropic action of inositol. Biochem. J. 48(4):448-452.

* 52. Best, C. H., C. C. Lucas, J. H. Ridout, and J. M. Patterson. 1950. Dose-response curves in the estimation of potency of lipotropic agents. J. Biol. Chem. 186:317-329.

53. Beveridge, J. M. R. 1944. Essential fatty acids and lipotropic action of inositol. Science 99(2583):539-540.

* 54. Beveridge, J. M. R., and C. C. Lucas. 1945. The effect of dietary fat on the lipotropic action of inositol. J. Biol. Chem. 157(1):311-321.

55. Binger, H. P. 1959. Vitamin and chemical studies on dehydrated forages. U.S. Dept. Agr. ARS 74-15, 2 pp.

56. Binkley, W. W., and M. L. Wolfrom. 1950. Chromatographic fractionation of cane blackstrap molasses and of its fermentation residue. J. Am. Chem. Soc. 72:4778-82.

* 57. Bly, C. G., F. W. Heggeness, and E. S. Nasset. 1943. Effects of pantothenic acid and inositol added to whole wheat bread on evacuation time, digestion and absorption in upper gastrointestinal tract of dogs. J. Nutrition 26:161-173.

58. Boehm, P., and G. Richarz. 1954. The quantitative estimation of inositol in phosphatides. Hoppe-Seyler's Zeitschr. Physiol. Chem. 298(3/5):110-120.

59. Bolene, C., J. Stamler, R. Harris, and L. N. Katz. 1949. Effect of choline and inositol on hypercholesterolemia and hyperlipemia in the cholesterol-fed chick. Fed. Proc. 8(1):13.

60. Bouveng, H., and B. Lindberg. 1955. Low-molecular carbohydrates in algae. VII. Investigation of Fucus spiralis and Desmarestia aculeata. Acta Chem. Scand. 9:168-9.

61. Bremner, H. J., and R. H. Clark. 1936. The influence of hormones and of inositol (bias) extracts of amylase activity. Trans. Roy. Soc. Canada. Sect. III 3(30):145-148.

62. Briggs, G. M., and F. S. Daft. 1955. Water-soluble vitamins. I. Vitamin B12, folic acid, choline, and p-aminobenzoic acid. Ann. Rev. Biochem. 24:339-92.

63. Broekhuyse, R. M. 1968. Changes in myoinositol permeability in the lens due to cataractous conditions. Biochim. Biophys. Acta 163(2):269-72.

64. Broquist, H. P. 1958. Water-soluble vitamins. I. Folic acid, B12 group, choline. Ann. Rev. Biochem. 27:285-312.

65. Brown, R. J., and R. F. Serro. 1953. Isolation and identification of O-alpha-D-galactopyranosylmyo-inositol and of myo-inositol from juice of the sugar beet (Beta vulgaris). J. Am. Chem. Soc. 75:1040-2.

66. Burns, J. J., and A. H. Conney. 1960. Water-soluble vitamins, Part I. (Ascorbic acid, nicotinic acid, vitamin B6, biotin, inositol). Ann. Rev. Biochem. 29:413-36.

67. Butturini, U., and A. Baronchelli. 1950. Synergism of action between alpha-tocopherol and inositol; clinico-experimental study. Rass. Fisiopat. Clin. 22:835-843.

68. Caldwell, M. L., N. Larson, and B. Huston. 1952. Absence of meso-inositol in purified amylase preparations from different sources. Cereal Chem. 29:463-9.

69. Calhoun, W. K., F. N. Hepburn, and W. B. Bradley. 1960. The distribution of the vitamins of wheat in commercial mill products. Cereal Chem. 37:755-61.

70. Calvery, H. O., J. H. Draize, and E. P. Lang. 1946. Metabolism and permeability of normal skin. Physiol. Rev. 26:495-540.

71. Campling, J. D., and D. A. Nixon. 1954. Inositol content of fetal blood and fetal fluids. J. Physiol. (London) 126:71-80.

72. Cantarelli, C. 1955. Microbiological determination of meso-inositol. Giorn. Biochim. 4:18-32.

73. Capello, M. A. 1954. Qualitative aspects of the anthrone test for carbohydrates. Rev. Assn. Bioquim. Argentina 18:41-3.

74. Capretti, G., and G. Paglia. 1950. Lipotropic factors and arteriosclerosis. Effect of methionine, choline, and inositol on cholesterol-induced arteriosclerosis. Giorn. Clin. Med. (Parma) 31:1120-37.

75. Carnovale, E., A. Fratoni, M. Lombardi, and C. Miuccio. 1964. Thiamine, riboflavine, inositol, and niacin in hard and soft Italian wheats. Quaderni Nutr. 24(2):163-85.

76. Carr, C. J., F. F. Beck, and J. C. Krantz, Jr. 1936. Sugar alcohols. 5. Chemical composition and sweet tarts. J. Am. Chem. Soc. 58:1394-95.

77. Carta, Q., and F. Pugliese. 1961. The action of inositol on the metabolism of carbohydrates. Congr. Federation Intern. Diabete, 4th, Geneva, Switz. 1:763-5.

78. Caspary, W. F., and R. K. Crane. 1970. Active transport of myo-inositol and its relation to the sugar transport system in hamster small intestine. Biochim. Biophys. Acta 203(2):308-16.

79. Castor, J. G. B. 1953. B-complex vitamins of musts and wines as microbial growth factors. Appl. Microbiol. 1:97-102.

80. Casu, B., and M. Reggiani. 1956. Determination of inositol and heparin sodium in solid phase by infrared spectroscopy. Il Farmaco (Pavia) Ed. Sci. 11:934-46.

81. Cavalcanti, A. C., and F. Levis. 1950. Fatty livers caused by phosphorus. Arch. Sci. Med. 90:529-41.

82. Cerbulis, J. 1955. Carbohydrates in cacao beans. II. Sugars in Caracas cacao beans. Arch. Biochem. and Biophys. 58(2):406-413.

83. Charalampous, F. C. 1959. Biochemical studies on inositol. V. Purification and properties of the enzyme that cleaves inositol to D-glucuronic acid. J. Biol. Chem. 234(2):220-227.

84. Charalampous, F. C., S. Bumiller, and S. Graham. 1958. Site of cleavage of myo-inositol by purified enzymes of rat kidney. J. Am. Chem. Soc. 80:2022.

85. Chargaff, E. 1955. Biochemistry of the inositols. Soc. Biol. Chemists, India 222-6.

86. Charlampowicz, Z., and W. Soblech. 1965. Changes in pyridoxine, nicotinic acid, and inositol content during technological processing (canning and freezing) of sweet corn. Zeszyty Probl. Postepow Nauk Rolniczych 53:115-16.

87. Charlampowicz, Z., and W. Sobiech. 1966. Thiamine, riboflavine, nicotinic acid, pyridoxine, cyanocobalamin, and inositol content of some vegetable and fruit products. Przem. Spozyw. 20(12):812-19.

88. Charonnat, R., and P. Lechat. 1952. Inhibition of the action of local anesthetics on the cornea of the rabbit by compounds containing hydroxyl groups. Ann. Pharm. Franc. 10:246-52.

89. Chauchard, P., H. Mazoue, and R. Lecoq. 1948. Influence of various vitamins on the nervous effects of adrenaline, acetylcholine, choline, and adrenochrome. Compt. Rend. Soc. Biol. 142:1346-8.

90. Cheldelin, V. H., and R. J. Williams. 1942. The B vitamin content of foods. Univ. Texas Pub. No. 4237:105-24.

* 91. Cheldelin, V. N., A. M. Woods, and R. J. Williams. 1943. Losses of B vitamins due to cooling of foods. J. Nutr. 26:477-485.

92. Climenko, D. R., and E. W. McChesney. 1942. Role of inositol and p-Aminobenzoic acid in normal lactation. Proc. Soc. Exp. Biol. and Med. 51(1):157-159.

93. Clouse, R. C. 1942. Compilation of recent data on mineral and vitamin values of food. J. Am. Dietet. Assoc. 18:553-61.

94. Cojocaru, C., and C. Focsa. 1965. Effect of the biotin, pantothenic acid, and inositol contents of molasses on the yield and quality of the bakers' yeast. Ind. Aliment. 16(7):354-9.

95. Colas, M. C., R. Perles, and P. Malangeau. 1959. Comparison of renal excretion of linear polyols and meso-inositol. Ann. Pharm. Franc. 17:260-9.

96. Colas, M. C., R. Perles, and M. C. Blayo. 1959. Renal tubular reabsorption of inositol. Compt. Rend. Soc. Biol. 153:1182-5.

97. Connor, J. B., and M. F. James. 1948. Choline deficiency in the baby pig. J. Nutrition 36(3):339-349.

* 98. Coots, R. H. 1959. Studies on the metabolism of myo-inositol in animals. Dissertation Absts. 19(7):1541-1542.

99. Cormack, D. V., et al. 1964. Relative humidity, inositol and the effect of radiations on air-dried micro-organisms. Nature (London) 201:1103-5.

100. Cotte, J., and B. Guillot. 1968. Methods of identifying some polyols and their derivatives. Particular application of paper chromatography. Gattefosse-SFPA, Notice No. OL 0072, 8 pp.

101. Coulson, C. B., and W. C. Evans. 1958. Paper chromatography and paper electrophoresis of phenols and glycosides. J. Chromatog. 1:374-9.

102. Courtois, J. E., R. Perles, and K. Todorovitch. 1956. Chromatographic characterization of the glycidic substances of cerebrospinal fluid. Ann. Biol. Clin. (Paris) 14:706-9.

103. Cunha, T. J., S. Kirkwood, P. H. Phillips, and G. Bohstedt. 1943. Effect of inositol upon rat alopecia. Proc. Soc. Exp. Biol. and Med. 54(2):236-238.

104. Curto, G. M. 1957. Effect of vitamin E and of the association of vitamin E and meso-inositol on the lipide content of cow milk. Zootec. e Vet. 12:410-23; Atti Soc. Ital. Sci. Vet. 11:312-14.

105. Dalgliesh, C. E., E. C. Horning, M. G. Horning, K. L. Knox, and K. Yarger. 1966. A gas-liquid chromatographic procedure for separating a wide range of metabolites occurring in urine or tissue extracts. Biochem. J. 101:792-810.

106. Dal Pozzo, A. 1965. Scherer's reaction on thin-layer chromatography. Boll. Chim. Farm. 104:55-7.

107. Dam, H. 1944. Studies on vitamin E deficiency in chicks. J. Nutrition 27(2):193-211.

108. Dam, H. 1944. Ineffectiveness of vitamin E in preventing cholesterol deposition in the aorta. J. Nutrition 28:289-95.

109. Dam, H. 1949. Nutritional exudative diathesis in chicks. Intern. Congr. Biochem., Abstrs. of Communs. 1st Congr., Cambridge, Engl., 61-2.

110. Dam, H., and J. Glavind. 1942. Factors influencing capillary permeability in the vitamin E deficient chick. Science 96(2488):235-236.

111. Darbre, A., and F. W. Norris. 1956. Vitamins in germination. Determination of free and combined inositol in germinating oats. Biochem. J. 64(3):441-446.

112. Das, N., and B. C. Guha. 1935. Conversion of inositol. I. Oxidation of inositol by rat tissue. Hoppe-Seyler's Z. Physiol. Chem. 231:157-60.

113. Daughaday, W. H., J. Larner, and E. Houghton. 1954a. The renal excretion of inositol in normal and diabetic human beings. J. Clin. Invest. 33:326-32.

114. Daughaday, W. H., and J. Larner. 1954b. The renal excretion of inositol by normal and diabetic rats. J. Clin. Invest. 33(8):1075-1080.

115. Davis, O., and Y. T. Oester. 1952. Experimental arteriosclerosis: inhibitory effects of ascorbic acid and inositol. Proc. Soc. Exptl. Biol. and Med. 81(1):284-286.

116. Dawson, R. M. C., and N. Freinkel. 1961. Distribution of free mesoinositol in mammalian tissues, including some observations on the lactating rat. Biochem. J. 78:606-10.

117. Deffner, G. G. J., and R. E. Hafter. 1960. Giant nerve fibers of the squid. III. Identification and quantitative estimation of free organic ninhydrin-negative constituents. Biochim. et Biophys. Acta 42:189-99.

118. Dernehl, C. U., J. K. Cline, H. G. Swann, and W. D. Collings. 1943. Effect of excess pantothenic acid, p-aminobenzoic acid and inositol on adaptation of rats to lowered atmospheric pressures. Texas Rep. Biol. & Med. 1:143-152.

119. DeWind, L. T., G. D. Michaels, and L. W. Kinsell. 1952. Lipid studies in patients with advanced diabetic atherosclerosis. Ann. Internal Med. 37(2):344-351.

120. Diaz, H. 1943. Confirmation of the presence of theobromine in yerba mate. Rev. Inst. Bacteriol. "Carlos G. Malbran" (Buenos Aires) 11:349-51.

121. Dilthey, W., W. Schommer, J. Thewalt, and S. Henkels. 1937. l-Inositol in red rose blossoms. XXVI. Pyrenium compounds. Hoppe-Seyler's Z. Physiol. Chem. 245:171-74.

122. Dioguardi, N., and A. Magnoni. 1951. Inositol and lipocaic do not increase the elimination of urinary trigonellin in a normal patient. Acta Vitaminol. 5:264-5.

123. Di Porto, A., and S. Maymone. 1953. Methyl metabolism in experimental intoxication from carbon tetrachloride. Behavior of urinary trimethylamine under the action of lipotropic factors and of vitamins B12 and B1. Policlinico (Rome), Sez. Med. 60:59-68.

124. Doisy, E. A., Jr., and B. C. Bocklage. 1949. Chronic toxicity of gamma-isomer of hexachlorocyclohexane in the albino rat. Proc. Soc. Exptl. Biol. Med. 71:490-3.

125. Doisy, E. A., Jr., and B. C. Bocklage. 1950. Inositol and the toxicity of four isomers of benzene hexachloride (hexachlorocyclohexane) for the rat. Proc. Soc. Exptl. Biol. Med. 74:613-16.

126. Dotti, L. B., W. C. Felch, and S. J. Ilka. 1951. Inhibiting effect of inositol on serum cholesterol and phospholipides following cholesterol feeding in rabbits. Proc. Soc. Exptl. Biol. Med. 78:165-71.

127. Drill, V. A. 1954. Lipotropic effects of vitamin B12 and other factors. Ann. New York Acad. Sci. 57(6):654-663.

* 128. Dubin, H. 1917. A note on the influence of inosite upon the excretion of phenol in the dog. J. Biolog. Chem. 28(2): 429-430.

129. Duchesne, D. D., and R. Bernard. 1953. Further studies on lipotropism in the domestic duck (white Pekin). Can. J. Med. Sci. 31:474-84.

130. Duff, R. B. 1953. The intraconversion of aldose and ketose sugars effected by alkaline impurities in the paper of a chromatogram. Chemistry & Industry 898-9.

131. Dupuis, Y., and P. Fournier. 1962. Conditions for maintenance of calcemia in the rat by means of exogenous factors. Compt. Rend. Soc. Biol. 156:237-9.

132. Dyatlovitskaya, E. V., V. V. Voronkova, and L. D. Bergel'son. 1962. Thin-layer chromatography of polyhydroxy compounds on cellulose. Dokl. Akad. Nauk SSSR 145:325-7.

133. Eagle, H., V. I. Oyama, M. Levy, and A. Freeman. 1956. Myo-inositol as essential growth factor for normal and malignant human cells in tissue culture. Science 123:845-847.

134. Eagle, H., V. I. Oyama, M. Levy, and A. E. Freeman. 1957. Myo-inositol as an essential growth factor for normal and malignant human cells in tissue culture. J. Biol. Chem. 226(1):191-206.

135. Eagle, H., et al. 1963. Failure of 10 congeners of myo-inositol to support or to inhibit the growth of a cultured human cell. Biochemistry (Wash) 2:1125-6.

136. Eilert, M. L., and L. R. Dragstedt. 1946. Lipotropic action of lipocaic: A study of the effect of oral and parenteral lipocaic and oral inositol on the dietary fatty liver of the white rat. Am. J. Physiol. 147(2):346-351.

137. Eisenberg, F., Jr., and A. H. Bolden. 1964. Reproductive tract as site of synthesis and secretion of inositol in the male rat. Nature 202(4932):599-600.

138. Ely, P. A. 1966. The placental transfer of hexoses and polyols in the guinea-pig, as shown by umbilical perfusion of the placenta. J. Physiol. (London) 184:255-71.

139. Eng, C. P., and J. F. Morgan. 1969. The release of nucleic acids and protein from ascites tumor cells incubated with glycerol, dimethylsulfoxide, and inositol. Can. J. Biochem. 47(9):871-876.

140. Engel, R. W. 1942. Relation of B vitamins and dietary fat to the lipotropic action of choline. J. Nutrition 24:175-85.

* 141. Ershoff, B. H. 1946. Effects of massive doses of p-aminobenzoic acid and inositol on reproduction in the rat. Proc. Soc. Exptl. Biol. Med. 63:479-80.

142. Ershoff, B. H., and H. B. McWilliams. 1943. Reproduction on purified rations containing sulfaguanidine. Proc. Soc. Exp. Biol. and Med. 54(2):227-228.

143. Etcheverry, M. A., A. A. Marchissio, and R. E. Mancini. 1948. Action of ultraviolet light on polysaccharides. Rev. Soc. Argentina Biol. 24:147-55.

144. Everson, G. J., and H. J. Souders. 1957. Composition and nutritive importance of eggs. J. Am. Dietetic Assoc. 33(12):1244-1254.

145. Farrell, L., and A. G. Lochhead. 1931. Accessory nutrient for osmophilic yeasts. II. Comparison of honey activated with biosinositol. Canadian J. Res. 5:539-543.

146. Faure, M., M. J. Morelec-Coulon, J. Marechal, and L. Leborgne. 1959. The phosphatidic acids of the liver. Bull. Soc. Chim. Biol. 41:101-9.

147. Feigl, F., and V. Gentil. 1955. Spot test detection of inositol. Mikrochim. Acta 1004-7.

148. Feigl, F., and C. C. Neto. 1956. Spot test for diketones and quinones based on catalytic effect. Anal. Chem. 28:397-9.

149. Felch, W. C., and L. B. Dotti. 1949. Depressing effect of inositol on serum cholesterol and lipide phosphorus in diabetics. Proc. Soc. Exptl. Biol. Med. 72:376-8.

150. Felch, W. C., J. H. Keating, and L. B. Dotti. 1952. The depressing effect of inositol on serum cholesterol and lipide phosphorus in hypercholesteremic myocardial infarct survivors. Am. Heart J. 44:390-5.

151. Feldheim, W., and E. Jarmatz. 1956. Occurrence of beta-carotene and the vitamin content of sea buckthorn berries. Vitamine u. Hormone 7:263-8.

152. Felice, F. De. 1951. Content of L-ascorbic acid and dehydroascorbic acid in certain organs of vitamin C-deficient guinea pigs treated with riboflavin. Boll. Soc. Ital. Biol. Sper. 27:216-17.

153. Fellin, E. 1952. Interference of streptomycin, inositol and guanidine on effect of bacteria. Gior. Batt. e Immunol. 44:337-340.

154. Fenton, P. F., G. R. Cowgill, M. A. Stone, and D. H. Justice. 1950. The nutrition of the mouse. VIII. Studies on pantothenic acid, biotin, inositol, and p-aminobenzoic acid. J. Nutrition 42:257-69.

155. Fiechter, A. 1955. Growth, respiration, and fermentation of the cultured wine yeast Polymorphus II. Ber. Schweiz. Botan. Ges. 65:5-46.

156. Fiesser, B. 1953. Vitamin C and yeast growth. Beitr. Biol. Pflanz. 30:33-60.

157. Finean, J. B., J. N. Hawthorne, and J. D. E. Patterson 1957. Structural and chemical differences between optic and sciatic nerve myelins. J. Neurochem. 1:256-9.

158. Fioramonti, M. C., J. C. Bryant, W. T. McQuilkin, V. J. Evans, K. K. Sanford, and W. R. Earle. 1955. The effect of horse serum residue and chemically defined supplements on proliferation of strain L clone 929 cells from the mouse. Cancer Research 15:763-6.

159. Fischer, H. O. L. 1946. Chemical and biologic relationships between hexoses and inositols. Harvey Lect.

160. Fleury, P., and M. Joly. 1937. Separation of inositol from glucose and its quantitative determination. J. Pharmac. Chim. (8)26(129):341-353, 397-407.

161. Fleury, P., and A. Recoules. 1948. Chemical determination of inositol. Compt. Rend. 227:691-3.

162. Fleury, P., and A. Recoules. 1949. Chemical determination of inositol in the presence of glucides. Bull. Soc. Chim. Biol. 31:256-64.

163. Fleury, P. 1951. Some points on the investigation and determination of the inositols. Bull. Soc. Chim. Biol. 33:1061-74.

164. Fleury, P. F., J. E. Courtois, and P. Malangeau. 1953. Utilization of paper strip chromatography for the separation of m-inositol and scyllitol and their identification. Bull. Soc. Chim. Biol. 35:537-40.

165. Fondovila, M. E., and H. Diaz. 1943. Presence of sucrose and inositol in yerba mate (Ilex paraguayensis St. Hill). Rev. D. Inst. Bact., Buenos Aires 11:449-454.

166. Fondovila, M. E., and H. Diaz. 1943. The presence of inositol and sucrose in yerba mate. Anal. Asoc. Quimica Argentina 31(159):32-37.

* 167. Forbes, J. C. 1943. Lipotropic action of inositol. Proc. Soc. Exp. Biol. and Med. 54(1):89-90.

168. Forgash, A. J. 1958. The effect of inositol on growth, survival, and maturation in Periplaneta americana. Ann. Entomol. Soc. Am. 51:406-9.

169. Forgash, A. J., and R. F. Moore. 1960. Dietary inositol requirement of Periplaneta americana (L.). Ann. Ent. Soc. Amer. 53(1):91-94.

170. Forgash, A. J. 1962. Failure of myo-inositol to prevent the growth-inhibiting effects of lindane in Periplaneta americana. J. Econ. Entomol. 55(3):308-312.

171. Fournier, P. 1956. Osteogenic activity of meso-inositol and the concept of growth factor. Compt. Rend. 243:182-4.

172. Fournier, P., and H. Susbielle. 1960. Influence of glucosamine, inositol, and mannitol on calcium utilization, calcemia, and development of the digestive tract. Compt. Rend. Soc. Biol. 154:308-11.

173. Franzen, H., and E. Keyssner. 1923. Chemical components of green plants. XXIX. Water soluble components of blackberry (Rubusfructicosus) leaves. Ztschr. f. Physiol. Ch. 129:309-19.

* 174. Freinkel, N., G. Antony, H. R. Williams, and B. R. Landau. 1970. Metabolism of myo-inositol in rabbit kidney and in man. Biochim. Biophys. Acta 210(3):425-433.

175. Friedman, M. 1960. Phosphatides in experimental atheroma. Am. J. Clin. Nutrition 8:318-20.

176. Fryer, J. J., and F. W. Norris. 1958. Occurrence of inositol in flour mill stocks. J. Sci. Food Agr. 9:396-401.

177. Fuehner, H. 1926. The laxative effect of sugar types. Festschrift Alexander Tschirch 30-35.

178. Fukui, S., Y. Tani, and T. Kishibe. 1955. The role of B vitamins in sake brewing. I. Effects of B vitamins on the growth of Saccharomyces sake. J. Fermentation Technol. (Japan) 33:1-6.

179. Fukui, S., Y. Tani, and T. Kishibe. 1955. The role of B vitamins in sake brewing. V. Variation of the contents of pantothenic acid, inositol, biotin, and vitamin B6 during the polishing process. J. Fermentation Technol. (Japan) 33:238-9.

180. Fuzii, K., F. Fuzikawa, and Y. Kudo. 1937. The components of Euphorbia humifusa, Willd. J. Pharmac. Soc. Japan 57:88-89.

181. Gadsden, E. L., C. H. Edwards, and G. A. Edwards. 1960. Paper chromatography of certain vitamins in phenol and butanol-propionic acid-water solvents. Anal. Chem. 32:1415-17.

182. Gaitonde, M. K., and M. Griffiths. 1966. A spectrophotometric method for the determination of microquantities of free inositol in biological material. Anal. Biochem. 15(3):532-535.

183. Galanos, D. S., and V. M. Kapoulas. 1965. Fractionation and identification of milk polar lipids; triester glycophospholipids. Biochim. Biophys. Acta 98(2):293-312.

184. Galliard, T., and J. N. Hawthorne. 1963. Metabolism of myoinositol in mammalian liver. Biochem. J. 88(2):38p.

185. Gallus, H. P. C., and A. C. Jennings. 1971. Compounds of low molecular weight in washed wheat gluten. Australian J. of Biological Sciences 24(4):825-828.

* 186. Gavin, G., and E. W. McHenry. 1941a. Inositol: a lipotropic factor. J. Biol. Chem. 139:485.

* 187. Gavin, G., and E. W. McHenry. 1941b. The effects of biotin upon fat synthesis and metabolism. J. Biol. Chem. 141:619-625.

* 188. Gavin, G., J. M. Patterson, and E. W. McHenry. 1943. Comparison of the lipotropic effects of choline, inositol and lipocaic in rats. J. Biol. Chem. 148:275-9.

189. Gephardt, M. C. 1947. Xanthomatous biliary cirrhosis: Lipid levels while receiving inositol. Ann. Internal Med. 26(5):764-769.

190. Gershbein, L. L., A. Miller, and J. Al-Wattar. 1964. Effect of water-soluble vitamins on rat diaphragm carbohydrate metabolism. Arch. Biochem. Biophys. 107(3):359-62.

191. Ghircoiasiu, M. N., and A. Maximinian. 1967. Effect of inositol on hepatic and tegumentary nucleic acids and cholesterol in Lacerta agilis. Stud. Univ. Babes-Bolyai, Ser. Biol. 1:133-6.

192. Ghircoiasiu, M., and A. Maximinian. 1969. Action of inositol on the heart of Testudo graeca. Stud. Univ. Babes-Bolyai, Ser. Biol. 14(1):131-6.

193. Ghiringhelli, C., L. Belli, and C. Borelli. 1955. Lipotropic agents and liver regeneration. Influence of meso-inositol. Arch. Sci. Med. 99:106-11.

194. Ghosh, A., F. Charalampous, and B. R. Sisony. 1960. Metabolic function of myo-insotol. I. Cytological and chemical alterations in yeast resulting from inositol deficiency. J. Biol. Chem. 235:2522-8.

195. Ghosh, A., et al. 1967. Changes in the respiratory and glycolytic behaviour of Saccharomyces carlsbergensis due to inositol deficiency. Biochim. Biophys. Acta 136:19-26.

196. Glass, R. L., and W. F. Geddes. 1960. Grain-storage studies. XXXI. Changes occurring in low-molecular-weight compounds in deteriorating wheat. Cereal Chem. 37:568-72.

197. Gluzinska, W., D. Ostaszewicz, and W. Rzedowski. 1967. Investigations of the nutrients content (biotin, inositol and pantothenic acid) in Polish molasses from the industry campaign of 1963/64 and 1964/65. Pr. Inst. Lab. Badaw. Przem. Spozyw. 17(3):89-101.

198. Gothoskar, S. S., D. V. Rege, and A. Sreenivasan. 1954. Biotin metabolism in microorganisms. IV. Nucleic acid synthesis. Indian J. Med. Research 42:599-604.

199. Graefe, G. 1953. Chemical constitution and biological importance of the inositols. Arzneimittel-Forsch. 3:304-9.

200. Graefe, G. 1958. Meso-inositol, a little-known B-complex vitamin. Zucker- u. Suesswarenwirtsch. 11:920-3, 971-3.

201. Graham, R. K., G. R. Skurray, and P. Caiger. 1970. Nutritional studies on yeasts during batch and continuous fermentation. I. Changes in vitamin concentrations. J. of the Institute of Brewing 76(4):366-71.

202. Granados, H., E. Aaes-Jorgensen, and H. Dam. 1949. Influence of certain nutrients on changes in adipose and dental tissues of vitamin E-deficient rats. Brit. J. Nutrition 3:320-34.

* 203. Greenwald, I., and M. L. Weiss. 1917. The fate of inositol fed to dogs. J. Biol. Chem. 31:1-14.

204. Gregoire, A. T., D. Gongsakdi, and A. E. Rakoff. 1962. The presence of inositol in genital tract secretions of the female rabbit. Fertility & Sterility 13(3):300-303.

205. Gregoire, J. A. 1969. Reconstituted dried milk. French Pat. 1,583,482.

206. Gregory, R. A. 1935. A modification of Young's method for the determination of inositol in animal tissues. Biochem. J. 29(12):2798-2802.

207. Guha, B. C., and N. Das. 1934. Biological oxidation of inositol. Current Sci. 3:157-58.

208. Haenel, H. 1953. Anti-metabolites. Pharmazie 8(11):891-897; (12):991-1005.

209. Haenel, H. 1956. Microbiological investigation of the factors of the B complex in foods and feeding stuff. Ernaehrungsforschung 1:533-40.

210. Hahn, L., and J. Fekete. 1953. Synthetic inhibitors of hyaluronidase. I. Demonstration of the high inhibitory power of some diphenylmethane and triphenylmethane derivatives. Acta Chem. Scand. 7:798-805.

211. Hall, W. K., and V. P. Sydenstricker. 1947. The production of methionine deficiency in the rat with low-casein diets. Arch. Biochem. 12:147-52.

212. Halver, J. E., and J. A. Coates. 1957. A vitamin test diet for long-term feeding studies. Prog. Fish-Culturist 19(3):112-118; Sport Fish Absts., 2(4):No. 1152.

* 213. Handler, P. 1946a. Dietary factors in the regulation of liver lipid concentration. J. Biol. Chem. 162:77-85.

* 214. Handler, P. 1946b. Factors affecting the occurrence of hemorrhagic kidneys due to choline deficiency. J. Nutr. 31:621-633.

215. Handler, P., and R. H. Follis, Jr. 1948. The role of thyroid activity in the pathogenesis of hepatic lesions due to choline and cystine deficiency. J. Nutrition 35:669-87

216. Hardinge, M. G., and H. Crooks. 1961. Lesser-known vitamins in foods. J. Am. Dietet. Assoc. 38:240-5.

217. Harper, A. E., W. J. Monson, D. A. Benton, and C. A. Elvehjem. 1953. The influence of protein and certain amino acids, particularly threonine, on the deposition of fat in the liver of the rat. J. Nutrition 50(3):383-393.

218. Harris, P. N., M. E. Krahl, and G. H. A. Clowes. 1947. p-Dimethylaminoazobenzene carcinogenesis with purified diets varying in content of cysteine, cystine, liver extract, protein, riboflavin, and other factors. Cancer Res. 7(3):162-175.

219. Hartmann, F. 1954. meso-Inositol as lipotropic substance. Naturwissenschaften 41:231-2.

220. Hartmann, F., and H. J. Gerth. 1955. Mode of action of meso-inositol as a lipotropic substance in the rat on a deficient diet according to Best. Naunyn-Schmiedebergs Arch. Exptl. Pathol. Pharmakol. 224:322-6.

221. Hartmann, F., R. Hertel, G. Schulze, and H. Wellmer. 1952. The lipotropic activity of vitamin E and cystine, methionine, inositol, and invert sugar in the carbon tetrachloride-poisoned rat. Arch. Exptl. Path. Pharmakol. 214:152-7.

222. Hartmann, F., F. Ruwe, and G. Schulze. 1953. The influence of methionine, inositol, and invert sugar on the lipide metabolism of carbon tetrachloride-poisoned dogs. Naunyn-Schmiedeberg's Arch. Exptl. Pathol. Pharmakol. 217:98-106.

223. Hasan, S. H., A. Kotaki, and K. Yagi. 1970. Studies on myoinositol: XI. Effect of myoinositol on serum lipoprotein metabolism of rats suffering from fatty liver. Vitamins (Kyoto) 41(2):76-80.

224. Hasan, S. H., et al. 1971. Studies on myoinositol. 8. The incorporation of 3 H-myoinositol into phosphatidyl-inositol of fatty liver. J. Vitaminol. (Kyoto) 17:159-62.

225. Hauschild, W. 1935. Components of mate. Mitt. Lebensmittelunters. Hyg. 26:329-50.

226. Hauser, G. 1965. Energy- and sodium-dependent uptake of inositol by kidney cortex slices. Biochem. Biophys. Res. Commun. 19(6):696-701.

227. Hauser, G., M. Arnold, and V. N. Finelli. 1966. The effect of the injection of 2-O,C-methylene myoinositol into rats on inositol metabolism in brain and kidney slices. Biochim. Biophys. Acta 116(1):125-32.

228. Hauser, G. 1969. Hormonal, metabolic, and efflux studies. Ann. N.Y. Acad. Sci. 165(Art. 2):630-45.

229. Hauser, G. 1969. myo-Inositol transport in slices of rat kidney cortex. I. Effect of incubation conditions and inhibitors. Biochim. Biophys. Acta 173(2):257-66.

230. Hauser, G. 1969. myo-Inositol transport in slices of rat kidney cortex. II. Effect of the ionic composition of the medium. Biochim. Biophys. Acta 173(2):267-76.

231. Hawkins, J. 1952. Amine oxidase activity of rat liver in riboflavin deficiency. Biochem. J. 51:399-404.

232. Hay, G. W., B. A. Lewis, and F. Smith. 1963. Thin-film chromatography in the study of carbohydrates. J. Chromatog. 1(4):479-86.

233. Hay, G. W., and F. Smith. 1962. The carbohydrates of beer. I. The neutral, low-molecular-weight components of commercial beer. Am. Soc. Brewing Chemists Proc. 127-31.

234. Hees, H., and C. Tropp. 1926. Fermentation of substituted carbohydrates by bacteria of the coli and lactis aerogenic group. Zentralblatt f. Bakter. u. Parasitenk. I. Abt. 100:273-84.

235. Hegsted, D. M., G. M. Briggs, R. C. Mills, C. A. Elvehjem, and E. B. Hart. 1941. Inositol in chick nutrition. Proc. Soc. Exp. Biol. and Med. 47(2):376-377.

236. Hellendoorn, E. W., A. P. de Groot, L. P. Mijll Dekker, P. Slump, and J. J. L. Willems. 1971. Nutritive value of canned meals. J. of the Am. Dietetic Assoc. 58(5):434-441.

237. Helleu, C. 1956. The presence of mesoinositol in royal jelly. Profession Med. 7/8. From Bee World. 41:110(1960).

238. Helleu, C. 1957. Urinary elimination of meso-inositol in relation to the diet. Bull. Soc. Chim. Biol. 39:633-40.

239. Hendrickson, R., and J. W. Kesterson. 1951. Citrus by-products of Florida. Commercial production methods and properties. Florida Agr. Expt. Sta. Bull. No. 487:5-56.

240. Herken, H., and D. Maibauer. 1954. Inositol in brain metabolism. Klin. Wochschr. 32:1113-14.

241. Herken, H., and D. Maibauer. 1954. Liberation of inositol in metabolism experiments with brain tissue. Naturwissenschaften 41:166.

242. Herken, H., D. Maibauer, and F. Weygand. 1957. On the metabolism of mesoinosite. Zeitschr. Naturforsch. 12b(8/9):598-599.

243. Herken, H., D. Maibauer, and F. Weygand. 1958. Metabolism and excretion of meso-inositol. Arch. Exptl. Pathol. Pharmakol. 233:301-10.

* 244. Herrmann, G. R. 1946. Inositol decholesterization in old hens. Proc. Soc. Exptl. Biol. and Med. 63(2):436-438.

245. Herrmann, G. R. 1947. Some experimental studies in hypercholesterolemic states. Exptl. Med. and Surg. 5:149-59.

* 246. Hesselbach, M. L., and D. Burk. 1944. Chemotherapeutic regression of malignant mouse tumors. Record Chem. Progress 5:37-46.

247. Hewitt, J. A., and D. B. Steabben. 1921. Fermentation of inositol. Biochem. J. 15:665-66.

248. Hewitt, J. A., and D. de Souza. 1921. The effect of i-inositol on an isolated frog heart. J. of Physiol. 54:CXIX.

249. Hiraoka, K., S. Takahashi, and K. Yamaguchi. 1957. The effect of inositol in experimental hypercholesterolemia of the chicken. Shionogi Kenkyusho Nempo 7:667-76.

250. Hirst, E. L., and J. K. N. Jones. 1949. Quantitative analysis of sugars by means of partition chromatography. III. Determination of sugars by oxidation with sodium periodate. J. Chem. Soc. 1659-62.

251. Hokin, M. R., and L. E. Hokin. 1954. Effects of acetylcholine on phospholipides in the pancreas. J. Biol. Chem. 209(2):549-558.

252. Hokin, L. E., and M. R. Hokin. 1958. Acetylcholine and the exchange of inositol and phosphate in brain phosphoinositide. J. Biol. Chem. 233(4):818-821.

253. Holm, V. 1959. Investigations and studies of the etiology and pathogenesis of disseminated sclerosis. Acta Psychiat, Neurol. Scand. 34:60-109.

254. Horner, W. H., and I. H. Thaker. 1968. The metabolism of scyllo-inositol in Streptomyces griseus. Biochim. Biophys. Acta 165(2):306-8.

255. Hough, L. 1950. Application of paper partition chromatography to the separation of the polyhydric alcohols. Nature 165:400.

256. House, H. L. 1954. Nutritional studies with Pseudosarcophaga affinis, a dipterous parasite of the spruce budworm, Choristoneura fumiferana. I. A chemically defined medium and aseptic-culture technique. Can. J. Zool. 32:331-41.

257. Howard, C. F., Jr. 1963. The catabolism of myoinositol-2-C14 by rat kidney homogenates and slices. Dissert. Abst. 24(6):2251-2252.

* 258. Howard, C. F., Jr., and L. Anderson. 1967. Metabolism of myo-inositol in animals: II. Complete catabolism of myo-inositol-C14 by rat kidney slices. Arch. Biochem. Biophys. 118(2):332-339.

259. Igarashi, H., K. Zama, and M. Katada. 1956. The phosphatide of aquatic animals. IV. The lecithin, cephalin, neutral fat, and unsaponifiable matter of the heart of a rorqual (Balaenoptera borealis). Nippon Nogei-kagaku Kaishi 30:111-15.

260. Instituto de Biologia y Sueroterapia S.A. 1965. Antibiotic and sulfonamide oral preparations. Span. Pat. 308,870.

261. Ito, Y., Y. Nakamura, and T. Uemura. 1955. Physiology of sake yeast. X. Nutritional requirements of the sake yeasts isolated from several sake breweries. J. Fermentation Technol. (Japan) 33:421-6.

262. Iyer, N. T. 1961. I: The synthesis of optically active derivatives of streptamine. II: Studies on a myo-inositol antagonist in mice. Dissertation Absts. 21(10):2878.

263. Jackson, R. S., C. F. Wilkinson, Jr., L. Meyers, M. S. Bruno, and M. R. Benjamin. 1955. An evaluation of the effect of choline and inositol on the clinical course and serum lipids in patients with angina pectoris. Ann. Internal Med. 42(3):583-594.

264. Jarboe, J. K., and A. F. Mabrouk. 1969. Water-soluble flavour precursors of beef. Free amino acids, sugars and organic acids in aqueous beef extracts. Abstracts of Papers, Am. Chem. Soc. 158:AGFD 7.

265. Johnstone, R. M., et al. 1967. Transport of myo-inositol in Ehrlich ascites cells. Biochim. Biophys. Acta 135:1052-5.

266. Kachan, V. I. 1968. Study of the vitamin nutrition of Bacillus polymyxa Ross., a polymyxin M producer. Mikrobiol. Zh. (KYYIV) 30(6):510-514.

267. Kanazawa, A. 1961. The vitamin B complex in marine algae. I. On vitamin contents. Kagoshima Daigaku Suisan Gakubu Kiyo 10:38-69.

268. Kaufmann, H. P., and C. V. Viswanathan. 1963. Thin layer chromatography of fats. X. The analysis of the metabolism of lipids. Fette, Seifen, Anstrichmittel 65:538-43.

269. Kean, E. L., and F. C. Charalampous. 1959. New methods for the quantitative estimation of myo-inositol. Biochim. et Biophys. Acta 36:1-3.

270. Kefford, J. F. 1970. Analytical problems with fruit products. Fruit World & Market Grower 71(4):14-20.

271. Keresztesy, J. C., D. Laszlo, and C. Leuchtenberger. 1946. Neutralization of inhibition of tumor growth. Cancer Res. 6:128-30.

272. Kik, M. C. 1945. Effect of milling, processing, washing, cooking, and storage on thiamine, riboflavin, and niacin in rice. Arkansas Agr. Expt. Sta. Bull. 458:60 pp.

273. Kik, M. C. 1955. Influence of processing on nutritive value of milled rice. J. Agr. Food Chem. 3:600-3.

274. Kimoto, K. 1952. Industrial organic analysis with ceric sulfate. Repts. Inst. Ind. Sci. Tokyo Univ. 3:20-55.

275. Kimura, M., M. Tohma, Y. Okazawa, and N. Murai. 1969. Quantitative determination of mono- and oligosaccharides in potato tubers by gas-liquid chromatography. J. Chromatogr. 41(1):110-12.

276. Kindl, H., et al. 1966. Cyclitols: biosynthesis, metabolism and occurrence. Fortschr. Chem. Organ Naturst. 24:149-205.

277. Kisch, B. 1947. Studies in biochemistry, dedicated to Professor Dr. Carl Neuberg for his 70th birthday. Exptl. Med. and Surg. 5(2/3):97-303.

278. Klein, F. 1967. Study of the distribution of inositol in the cortex and nucleus of the calf lens. Experientia 23:202.

279. Koegl, F., and W. Van Hasselt. 1936. Isolation of Bios I (mesoinositol) from yeast. 21 plant growth substances. Hoppe-Seyler's Z. Physiol. Chem. 242:74-80.

280. Kojima, Y., and H. Kusakabe. 1954a. Isolation of natural substances by ion-exchange resins. I. Isolation of inositol, taurine, and succinic acid from the kidney of pig. J. Sci. Research Inst. (Tokyo) 48:121-5.

281. Kojima, Y., and H. Kusakabe. 1954b. Isolation of natural substances by ion-exchange resins. II. The content of inositol in the brain of pig. J. Sci. Research Inst. (Tokyo) 48:284-6.

282. Kolmark, G. 1956. Mutagenic properties of esters of inorganic acids investigated by the Neurospora back-mutation test. Compt. Rend. Trav. Lab. Carlsberg, Ser. Physiol. 26:205-20.

283. Koops, J. 1963. Cold storage defects of butter. Verslag. Ned. Inst. Zuivelonderzoek No. 80:193 pp.

284. Korotchenko, N. I., V. A. Bendosenko, and N. K. Koneva. 1970. Microbiological quantitative determination of inositol in yeasts. Prikl. Biokhim. Mikrobiol. 6(2):243-7.

285. Korting, G. W., and R. Schmitz. 1952. The influence of lipotropic and thyrostatic factors on fat deposition in the skin. Die Medizinische 26:116-18.

* 286. Kotaki, A., K. Natsume, Y. Yamamoto, and K. Yagi. 1966. Myoinositol. II. Possible role of dietary myoinositol on the growth promotion of young rats. J. Vitaminol. (Kyoto) 12(1):1-9.

287. Kotaki, A., T. Sakurai, and K. Yagi. 1967. Studies on myoinositol: VII. On the fate of injected myoinositol 3H in young rats. Vitamins (Kyoto) 35(3):208-212.

288. Kotaki, A., T. Sakurai, M. Kobayashi, and K. Yagi. 1967. Studies on myoinositol: (VIII). Effect of myoinositol on the cholesterol metabolism of rats suffering from experimental fatty liver. Vitamins (Tokyo) 36(3):231-236.

289. Kotaki, A., M. Okumura, Y. Nakagawa, and K. Yagi. 1968. Riboflavine tetranicotinate. V. Effect of riboflavine tetranicotinate on the hepatic and the serum lipoperoxide level of rats with fatty liver. Bitamin 37(5):466-71.

290. Kotaki, A., T. Sakurai, M. Kobayashi, and K. Yagi. 1968. Myoinositol. IV. Effect of myoinositol on the cholesterol metabolism of rats with experimental fatty liver. J. Vitaminol. (Kyoto) 14(2):87-94.

291. Kotaki, A., M. Okumura, S. H. Hasan, and K. Yagi. 1969. Studies on myoinositol: X. Effect of myoinositol on the prevention of fatty liver induced by orotic acid. Vitamins (Kyoto) 40(4):219-223.

292. Koyama, Y., and H. Iizuka. 1960. Royal jelly. I. The vitamin content in royal jelly. J. Japan Soc. Food Nutrition 13(2):50-2.

293. Krause, A. C., and R. Weekers. 1938. Inositol in ocular tissues. Arch. Ophth. 20:299-303.

294. Krebs, H. A., and P. Lund. 1966. Formation of glucose from hexoses, pentoses, polyols, and related substances in kidney cortex. Biochem. J. 98(1):210-14.

295. Krehl, W. A., and G. R. Cowgill. 1950. Vitamin content of citrus products. Food Res. 15(3):179-191.

296. Krehl, W. A., and G. R. Cowgill. 1955. Nutrient content of cane and beet sugar products. Food Res. 20(5):449-468.

297. Kreitmaier, G. 1952. Effect of B vitamins and amino acids on the rate of multiplication of Paramecium caudatum. Z. Vitamin-, Hormon- u. Fermentforsch. 4:542-554.

298. Kryukova, N. N. 1947. Inositol in tea leaves. Biokhimiya 12:516-23.

299. Kubena, L. F. 1971. The effects of certain dietary factors on the elimination of various chlorinated hydrocarbons. Dissertation Abstracts International. Section B. The Sciences and Engineering 32(1)2: Order No. 71-17814.

300. Kumagawa, H. 1922. Decomposition of meso-inositol and glycerin in the manner of the real sugars by Bacillus lactis aerogenes. Biochem. Ztschr. 131:157-60.

301. Kurasawa, H., T. Hayakawa, and Y. Kanauchi. 1968. Biosynthesis of myoinositol in rice seeds. I. Microbiological assay of myoinositol in rice seeds by Schizosaccharomyces pombe. Nippon Nogeikagaku Kaishi 42(10):587-590.

302. Kursanov, A., N. Kriukova, and E. Vyskrebentsova. 1948. Inositol as an intermediary product in the transformation of sugars into polyphenols. Biokhimiia 13(6):530-537.

303. Kursanov, A. L., M. Vorob'eva, and E. Vyskrebentseva. 1949. meso-Inositol in tea leaves and its formation paths. Doklady Akad. Nauk SSSR 68:737-40.

304. Kuttner, R. E., A. B. Lorincz, and A. Portoghese. 1967. Urinary amino acids of rats receiving high sugar diets. J. Nutr. 91(4):514-20.

305. Laird, R. D., et al. 1971. Lipotropic activity of inositol and chlortetracycline alone and in various combinations of choline, vitamin B12 and folic acid. Activity of three liver extracts with assays for these substances. Arch. Int. Pharmacodyn. Ther. 194:103-16.

306. Lambou, M. G. 1956. Multiple spray technique for locating sugars and polyols on the same chromatogram. Anal. Chem. 28:1216.

307. Lance, B. G., and A. G. Hogan. 1948. Vitamin Bc, inositol, nicotinic acid in the nutrition of the turkey. J. Nutrition 36:369-79.

308. Lanciano, O., and P. Magnanelli. 1954. The action of inositol and rutin on the modification of the capillariscope picture produced by hyaluronidase. Boll. Soc. Ital. Biol. Sper. 30:972-4.

309. Lane, R. L., and R. J. Williams. 1948. Inositol, an active constituent of pancreatic (alpha) amylase. Arch. Biochem. 19:329-35.

* 310. Laszlo, D., and C. Leuchtenberger. 1943. Inositol a tumor growth inhibitor. Science 97(2527):515.

311. Launer, H. F., and Y. Tomimatsu. 1953. Rapid accurate determination of carbohydrates and other substances with the dichromate heat-of-dilution method. Anal. Chem. 25:1767-1769.

312. LeBaron, F. N., et al. 1963. The amounts and distribution of free inositol and free and protein-bound phosphoinositides in brain tissues. J. Neurochem. 10:677-83.

313. Lecoq, R. 1948. Effects of vitamin E and linoleic acid on acid-base balance of the blood when injected intravenously. Compt. Rend. Soc. Biol. 142:286-8, 288-290.

314. Lecoq, R. 1950. Lipotropic factors from the biological and experimental points of view. Rev. Path. Comparee et Hyg. Gen. 50:119-24.

315. Lecoq, R., P. Chauchard, and H. Mazoue. 1948. Disturbances in neuromuscular excitability in the course of alimentary disturbances and avitaminoses. XXV. - Vitamin requirements related to lipid and cholesterol metabolism. Bull. Soc. Chim. Biol. 30(5/6):296-305.

316. Lecoq, R., P. Chauchard, and H. Mazoue. 1949. Chronaximetric assay of vitamins in milk. Rev. Path. Comparee et Hyg. Gen. Hyg. 49:468-76.

317. Lecoq, R., P. Chauchard, and H. Mazoue. 1951. Influence of substances producing acidosis or alkalosis on the effect of vitamins. Ann. Pharm. Franc. 9:711-18.

318. Lembach, K., et al. 1967. Metabolic functions of myo-inositol. VI. Impairment of amino acid transport in KB cells caused by inositol deficiency. J. Biol. Chem. 242:2606-14.

319. Lendle, A. 1938. Substances in pumpkin (gourd) seeds. Arch. Pharmaz. Ber. Dtsch. Pharmaz. Ges. 276:45-53.

320. Leconian, L. H., and V. G. Lilly. 1945. The comparative value of different test organisms in the microbiological assay of B vitamins. West Virginia Agr. Expt. Sta. Bull. 319:35 pp.

321. Letham, D. S. 1963. Isolation of a kinin from plum fruitlets and other tissues. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) No. 123:109-17.

322. Levy, M. 1957. Action of choline and inositol on the metabolic disorders observed with animals presenting different types of steatoses. Arch. Sci. Physiol. 11:157-67.

323. Lewin, L. M. 1959. Inositol phosphatide fraction obtained from green peas. Univ. Microfilms L.C. Card No. Mic 59-5309, 117 pp; Dissertation Abstr. 20:1564-5.

324. Lewin, L. M. 1967. The role of myo-inositol in cellular metabolism. Georgetown Med. Bull. 20(3):131-6.

325. Lewin, L. M., and A. C. Wagenknecht. 1960. Inositol phosphatide of peas. Arch. Biochem. Biophys. 87:239-46.

326. Lichstein, H. C., H. A. Waisman, K. B. McCall, C. A. Elvehjem, and P. F. Clark. 1945. Influence of pyridoxine, inositol, and biotin on susceptibility of Swiss mice to experimental poliomyelitis. Proc. Soc. Exptl. Biol. and Med. 60(2):279-284.

327. Lilyblade, A. L., and D. W. Peterson. 1962. Inositol and free sugars in chicken muscle post-morten. J. Food Sci. 27:245-9.

328. Lin, C. R. 1957. Effect of choline, p-aminobenzoic acid, and inositol on the growth of mice. Bitamin 13:508-513.

329. Lindley, D. C., and T. J. Cunha. 1946. Nutritional significance of inositol and biotin for the pig. J. Nutrition 32(1):47-59.

330. Lino, A. 1952. The action of lipotropic factors on hepatic steatosis caused by biotin. Congr. Intern. Biochim., Resumes Communs., 2e Congr., Paris 163.

331. Littman, M. L., et al. 1964. Effect of water soluble vitamins and their analogues on growth of Candida albicans. III. Para-aminobenzoic acid, nicotinic acid, inositol and their analogues. Mycopathologia 22:117-36

332. Lornitzo, F. A. 1968. A method for colorimetric assay of inositol and some of its phosphate derivatives. Anal. Biochem. 25(1-3): 396-405.

333. Luckey, T. D. 1954. Single diet for all living organisms. Science 120:396-8.

334. Lueers, H., and K. Silbereisen. 1927. The phytase of malt. Wchschr. Brauerei 44:263-68, 273-278.

* 335. MacFarland, M. L., and E. W. McHenry. 1945. The dietary production of fatty livers resistant to the action of choline. J. Biol. Chem. 159:605-9.

336. MacFarland, M. L., and E. W. McHenry. 1948. Further observations on the lipotropic need for inositol. J. Biol. Chem. 176:429-34.

337. Magasanik, B. 1955. The metabolic control of histidine assimilation and dissimilation in Aerobacter aerogenes. J. Biol. Chem. 213:557-69.

338. Maier, V. P., and F. H. Schiller. 1959. Progress of chemical studies of Deglet Noor dates. Date Growers Inst., Coachella Valley, Calif., Rept. 36:8-10.

339. Malangeau, P. 1958. Inositol and scyllitol in food plants. Qualitas Plant. et Materiae Vegetabiles 3-4:393-404.

340. Malangeau, P. 1956. The cyclohexitols (meso-inositol and scyllitol) present in human urine. Bull. Soc. Chim. Biol. 38:729-41.

341. Malangeau, P. 1956. Characterization of the cyclohexitols in some human and animal tissues. Bull. Soc. Chim. Biol. 38:1003-8.

342. Malangeau, P., and A. L. Jouannet. 1954. Nature of the cyclohexitols present in the urine of mammals. Bull. Soc. Chim. Biol. 36:381-3.

343. Mann, T. 1951. Inositol, a major constituent of the seminal vesicle secretion of the boar. Nature 168:1043-4.

344. Mann, T. 1952. Inositol and ergothioneine, two new constituents of seminal plasma. Congr. Intern. Biochim., Resumes Communs., 2e Congr., Paris 365-6.

345. Marfori, L., S. Vulterini, and N. Napoleone. 1950. Experimental atherosclerosis. II. Lipide fractions of the blood serum of rabbits fed a hypercholesterolic diet and treated with lipotropic substances. Rass. Fisiopatol. Clin. e Terap. (Pisa) 22:31-40.

346. Marfori, L., S. Vulterini, and S. Teodori. 1950. Experimental atherosclerosis. VI. Preventive action of some lipotropic substances on aortic atheromasia. Sperimentale 101:132-5. Ann. Igiene 60:31.

347. Marolla, I. 1960. Mesoinositol as a substance sparing vitamin C. Boll. Soc. Ital. Biol. Sper. 36:860-3.

* 348. Martin, G. J. 1942. The interrelationship of p-aminobenzoic acid and inositol. Am. J. Physiol. 136(1):124-127.

* 349. Martin, G. J., M. R. Thompson, and J. de Carvaljal-Forero. 1941. The influence of inositol and other B complex factors upon the motility of the gastro-intestinal tract. Am. J. Digest. Dis. 8(8):290-295.

350. Martini, V. 1955. The serum protein picture during regeneration of the liver in rats treated with lipotropic substances. Boll. Soc. Ital. Biol. Sper. 31:742-744.

351. Maruyama, G. M., and P. H. Phillips. 1948. Reproduction and lactation studies with rats fed natural and purified rations. J. Nutrition 36(5):613-623.

352. Masamune, H., H. Kawasaki, and Y. Shinohara. 1960. Anemia-inducing substances from stomach cancer tissue. I. On a dialyzable active substance. Tohoku J. Exptl. Med. 72:348-55.

* 353. Maurer, H., and H. Seckfort. 1953. The effect of inositol on basal metabolism. Klin. Wochschr. 31:911-12.

354. McDonald, R. E. 1970. Some effects of post harvest handling methods on sugars and inositol in the sweet potato (Ipomoea batatas Poir). Dissertation Abstracts International. Section B. The Sciences & Engineering 31(4)1637:Order No. 70-18546.

355. McDonald, R. E., and D. W. Newson. 1970. Extraction and gas-liquid chromatography of sweet potato sugars and inositol. J. Am. Soc. Hort. Sci. 95(3):299-301.

356. McHenry, E. W., and J. M. Patterson. 1944. Lipotropic factors (including choline, lipocaic and inositol). Physiol. Rev. 24:128-167.

357. McIntire, J. M., L. M. Henderson, B. S. Schweigert, and C. A. Elvehjem. 1943. Growth of rats on synthetic diets containing limited amounts of thiamine. Proc. Soc. Exptl. Biol. Med. 54:98-100.

358. McKay, H., et al. 1943. The effect of vitamin D on calcium retentions. J. Nutrition 26:153-9.

* 359. McKibbin, J. M., and D. Brewer. 1953. Effect of inositol feeding on the inositol phosphatides and other lipids of human blood plasma. Proc. Soc. Exptl. Biol. and Med. 84(2):386-388.

360. Meinke, W. W., B. R. Holland, and W. D. Harris. 1949. Solvent extraction of rice bran. Production of B-vitamin concentrate and oil by isopropanol extraction. J. Am. Oil Chemists' Soc. 26:532-4.

361. Melampy, R. M., and R. B. Mason. 1957. Androgen and the myo-inositol content of male accessory organs of the rat. Proc. Soc. Exptl. Biol. Med. 96:405-8.

362. Menzinsky, G. 1950. On factors influencing the metabolism and growth of Saccharomyces cerevisiae (top yeast) under aerobic conditions. Ark. Kemi. 2(1):1-94.

363. Metcalf, R. L. 1947. Relative toxicities of isomeric hexachlorocyclohexanes and related materials to thrips. J. Econ. Entomol. 40:522-5.

364. Metzenberg, R. L., and H. K. Mitchell. 1954. Detection of periodate-oxidizable compounds on paper chromatograms. J. Am. Chem. Soc. 76:4187.

365. Meyer, A. E. 1946. Inositol, a constituent of thyroid gland, effect on perfused rabbit heart. Proc. Soc. Exptl. Biol. Med. 62:111-12.

366. Michell, R. H., J. N. Hawthorne, R. Coleman, and M. L. Karnovsky. 1970. Extraction of polyphosphoinositides with neutral and acidified solvents: A comparison of guinea-pig brain and liver and measurements of rat liver inositol compounds which are resistant to extraction. Biochim. Biophys. Acta 210(1):86-91.

367. Middelhoven, W. J. 1969. The effect of myo-inositol in the synthesis of arginase and ornithine transaminase in baker's yeast. Biochim. Biophys. Acta 192(2):243-251.

368. Milhorat, A. T., and W. E. Bartels. 1945. The defect in utilization of tocopherol in progressive muscular dystrophy. Science 101(2613):93-94.

369. Mishkin, A. R., R. S. Bower, and L. E. Anderson. 1970. Isolation of myo-inositol from roasted Coffea arabica. Carbohyd. Res. 13(1):170-2.

370. Miyake, S. 1922. Chemical studies of maize pollen. I. Isolation of phytosterin and inositol. J. Biochem. 2:27-32.

371. Moldawski, B. 1925. Inositol in pepsin. J. Russ. Phys.-Chem. Ges. 57:13-16.

372. Morrison, L. M. 1953. Lipogram studies in coronary atherosclerosis. I. Response to lipotropic agents. Angiology 4(2):123-129.

* 373. Moscatelli, E. A., and J. Larner. 1959. The metabolism in the rat of photosynthetically prepared myo-inositol-C14. Arch. Biochem. and Biophys. 80(1):26-34.

374. Moses, C., G. L. Rhodes, and A. Delacio. 1952. The effect of large doses of inositol on experimental atherosclerosis. Angiology 3(3):238-240.

375. Mosonyi, L., L. Pollak, R. Zulik, and G. Karolyhazi. 1956. Streptomycin and endocrine system. Experientia 12:311-13.

376. Mosonyi, L. 1962. Influence of basic antibiotics on human enzymes. Arzneimittel-Forsch. 12:591-7.

377. Murray, M. R., H. M. DeLam, and E. Chargaff. 1951. Specific inhibition by meso-inositol of colchicine effect on rat fibroblast. Exptl. Cell Research 2:165-77.

378. Nagai, Y., and Y. Kimura. 1958. Detection of inositol and inositol diphosphate on paper chromatograms. Nature 181:1730-1.

379. Nakanishi, S., and T. Tsukahara. 1954. The effect of vitamins on the growth of sake yeast. J. Soc. Brewing (Japan) 49:471-5.

380. Narumi, K., M. Arita, and M. Kitagawa. 1969. Gas chromatographic analysis of free myo- and scyllo-inositols in animal tissues. Jap. J. Exp. Med. 39(4):399-407.

381. Nath, M. C., and A. Saikia. 1959. Effects of essential fatty acids, inositol, vitamin B12, and hydrolyzed glucose cyclo-acetoacetate on blood coagulation factors in rabbits exhibiting hyperlipemia induced by feeding saturated fat. J. Nutrition 69:403-11.

382. Nath, M. C., and A. Saikia. 1959. Physiological significance of inositol, vitamin B12 and glucose cycloacetoacetate in cholesterol-induced atherosclerosis on rabbits. Arch. Biochem. and Biophys. 79:216-223.

* 383. Natsume, K. 1965. Myoinositol. III. Effect of an excess dosage of myoinositol on the pattern of lipids in the liver of young rats. Bitamin 32(4):363-8.

384. Needham, J. 1923. Inositol studies. I. A quantitative determination method. Biochemical J. 17:422-29.

385. Neidhardt, F. C., and B. Magasanik. 1957. Effect of mixtures of substrates on the biosynthesis of inducible enzymes in Aerobacter aerogenes. J. Bacteriol. 73:260-3.

386. Nelson, E. K., and G. L. Keenan. 1933. Inactive inositol in citrus fruits. Science, New York 77:561.

387. Nicrosini, F., and O. Piccinelli. 1960. Various drugs acting on the serum lipid pattern of atherosclerosis. Therapeutic significance of an extractive heparinoid. Drugs Affecting Lipid Metab., Proc. Symp., Milan 508-11.

388. Nielsen, E., and A. Black. 1944. Role of inositol in alopecia of rats fed sulfasuxidine. Proc. Soc. Exp. Biol. and Med. 55(1):14-16.

389. Nixon, D. A. 1959. Inositol concentration in the cerebrospinal and ocular fluids and tissues of the fetal and adult sheep. Nature 184(Suppl. 12):906-7.

390. Nixon, D. A. 1960. Metabolism of meso-inositol-C14 in the rat. Nature 187:77-9.

391. Nixon, D. A. 1968. The concentration of free meso-inositol in the plasma of perfused sheep foetuses. Biol. Neonat. 12:113-20.

392. Norris, F. W., and A. Darbre. 1956. Microbiological assay of inositol with a strain of Schizosaccharomyces pombe. Analyst 81:394-400.

393. Norris, L. C., and G. F. Heuser. 1945. Water-soluble vitamins. Ann. Rev. Biochem. 14:469-524.

394. Northam, B. E., and F. W. Norris. 1952. A microbiological assay of inositol: its development and statistical analysis. J. Gen. Microbiol. 7:245-56.

395. Odincova, E. N., and L. A. Gevorkyan. 1969. The vitamin content of grapes and wine musts. Biologicheskii Zhurnal Armenii 22(9):96-97.

396. Odintsova, E. N., and E. A. Gevorkyan. 1968. Content of B vitamins in grapes. Vinodel. Vinograd. SSSR 28(8):50-2.

397. Oggioni, G. 1953. The antiscorbutic action of inositol. Boll. Soc. Ital. Biol. Sper. 29:1421-2.

398. Okochi, N., and T. Egawa. 1941. Bios. III. Synthesis of sodium dl-pantothenate. J. Agr. Chem. Soc. Japan 17:578-80.

399. Okoti, N. 1938. Studies on Bios (inositol). I. The action of beta-alanin, mesoinositol, vitamin B1, B2, and B6 on Saccharomyces cerevisiae. Bull. Agric. Chem. Soc. Japan 14:122-23.

400. Okuda, T., and K. Konishi. 1968. Coriose and related compounds. I. Isolation of coriose and co-occurring carbohydrates from leaf, stem, and fruit of coriaria japonica. Yakugaku Zasshi 88(10):1329-1332.

401. Okuzono, H. 1956. Fatty liver. II. Ethionine-induced fatty liver. Kumamoto Med. J. 9:1-7.

402. Oliver, G. L., et al. 1968. Inositol in normal lenses and cataracts. Canad. J. Ophthal. 3:266-9.

403. Oomori, I., K. Sumino, Y. Tani, and S. Fukui. 1968. Physiological and biochemical studies on Saccharomyces sake. X. Contents of inositol and inositol phosphatides and their subcellular distribution in Saccharomyces sake. Hakko Kogaku Zasshi 46(2):92-8.

404. Orsini, S. 1957. Inositol and morphine spasm. Vitaminologia (Turin) 15:165-70.

405. Ostrowski, W. 1954. Microbiological methods for the assay of vitamins and amino acids. Acta Microbiol. Polon. 3:35-46.

406. Ournac, A. 1970. Vitamins of wine. A review. Annales de la Nutrition et de l'Alimentation 24(3):B333-65.

407. Park, C. 1968. Histochemical studies of rat salivary gland phosphatase and esterase to determine the effect of inositol. Ch'oesin Uihak 11(5):103-6.

408. Partridge, S. M., and R. G. Westall. 1948. Filter-paper partition chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apple juice, egg white, and fetal blood of sheep. Biochem. J. 42:238-50.

409. Pavcek, P. L., and H. M. Baum. 1941. Inositol and spectacled eye in rats. Science 93:502.

410. Pearson, W. N., and W. J. Darby, Jr. 1967. Catabolism of 14C-labeled thiamine by the rat as influenced by dietary intake and body thiamine store. J. Nutr. 93(4):491-498.

411. Perles, R., and M. C. Colas. 1957. Urinary elimination of meso-inositol in diabetic subjects. Compt. Rend. Soc. Biol. 151: 1829-30.

412. Perles, R., and M. C. Colas. 1958. Excretion of mesoinositol in urine in some diseases. Bull. Soc. Chim. Biol. 40:913-21.

413. Perles, R., M. C. Colas, and M. C. Blayo. 1960. Mechanism of the renal excretion of inositol in the dog. Rev. Franc. Etudes Clin. et Biol. 5(1):31-36.

414. Perles, R., and M. C. Colas. 1960. Microbiological determination of blood mesoinositol; normal and pathological variations. Clin. Chim. Acta 5:95-9.

415. Peynaud, E., and S. Lafourcade. 1954. Recent studies on growth factors at the Enology Station of Bordeaux. 10th Congr. Intern. Inds. Agr. Y Aliment., Madrid. 1678-87.

416. Peynaud, E., and S. Lafourcade. 1955. Inositol in grapes and wine. Its microbiological dosage. Ann. Inst. Natl. Recherche Agron. Ser. E. 4:381-96.

417. Pfiffner, J. J., and O. D. Bird. 1956. Water-soluble vitamins. I. Vitamin B12, folic acid, choline, and p-aminobenzoic acid. Ann. Rev. Biochem. 25:397-434.

418. Pittoni, A., and C. R. Rossi. 1954a. Mechanism of action of lipotropic agents. III. Influence of choline on the intermediate metabolism of lipides in the liver. Arch. Sci. Biol. (Italy) 38:26-32.

419. Pittoni, A., and C. R. Rossi. 1954b. Mechanism of action of lipotropic factors; hepatic lipid metabolism, methionine, inositol. Arch. Sc. Biol. 38:47-55.

* 420. Platt, B. S., and G. E. Glock. 1943. The estimation of inositol in animal tissues. Biochem. J., 37:709-12.

421. Pomeranz, Y., M. J. Carvajal, M. D. Shogren, R. C. Hoseney, and K. F. Finney. 1970. Wheat germ in breadmaking. II. Improving breadmaking properties by physical and chemical methods. Cereal Chem. 47(4):429-37.

422. Pora, E. A., and M. Ghircoiasiu. 1958. The action of inositol on the hair of white rats. Acad. Rep. Populare Romine, Filiala Cluj, Studii Cercetari Biol. 9:97-101.

423. Pora, E. A., M. Ghircoiasiu, and T. Perseca. 1963. Influence of inositol on the fixation of 45Ca in the chicken bones. Studia Univ. Babes-Bolyai, Ser. Biol. 8(1):121-3.

424. Porcellati, G. 1967. Incorporation of 14C-choline and tritiated inositol into rat adipose tissue phospholipid in vitro. Nature (London) 215(5106):1189-1190.

425. Posternak, T., W. H. Schopfer, and J. Deshusses. 1960. Research on the functions and metabolism of meso-inositol. II. Comparative study of the phospholipides of Schizosaccharomyces pombe. Arch. Sci. (Geneve) 13(4):530-537.

426. Posternak, T., W. H. Schopfer, and D. Reymond. 1955. Biochemistry of cyclitols I. Metabolism of meso-inositol in the rat. Helv. Chim. Acta 38:1283-8.

427. Pratt, R., et al. 1966. Production of pantothenic acid and inositol by Chlorella vulgaris and C. pyrenoidosa. J. Pharm. Sci. 55: 799-802.

428. Preece, I. A. 1962. Phytin. Brewers Digest 37:59-62.

429. Pritchard, H., and D. R. Wraige. 1953. Vitamin B group in fish meal. J. Sci. Food Agr. 4:172-6.

430. Pritchard, H. 1953. Selection of methods for routine assays for members of the vitamin-B complex. Analyst 78:460-6.

431. Promayon, J. 1966. Lipids of solvent-extracted peanut oil cake. Oleagineux 21(11):683-6.

432. Rabbi, A. 1954. Dietary factors and experimental liver lesions. Minerva Med. II:371-6.

433. Radhakrishnamurty, R., and P. S. Sarma. 1953. Paper chromatographic separation of B-group vitamins. Current Sci. (India) 22:209-10.

434. Rajagopalan, K. V., and P. S. Sarma. 1956. Investigation of shark liver residue. Determination of B-vitamins and preparation of proteolyzed extracts. J. Sci. Ind. Research (India) 15C:227-31.

435. Rajalakshmi, S., V. Srinivasan, and P. S. Sarma. 1960. Accumulation of cholesterol in inositol deficiency. Proc. Soc. Exptl. Biol. Med. 104:97-9.

436. Rakieten, M. L., B. Newman, K. G. Falk, and I. Miller. 1952. Comparison of some constituents in fresh-frozen and freshly squeezed orange juice. J. Am. Dietect. Assoc. 28:1050-3.

437. Ralli, E. P., and S. H. Rubin. 1942. The effect of meat and meat fractions on the fatty liver of the depancreatized and pancreatic-duct ligated dog. Am. J. Physiol. 138(1):42-49.

438. Ramachandran, S., and P. S. Sarma. 1954. Role of inositol in the activity of alpha-amylase. Indian J. Med. Res. 42(2):201-209.

439. Ramachandran, S., R. Sivaramakrishnan, and P. S. Sarma. 1956. Further studies on the role of inositol in the activity of amylase. Indian J. Med. Research 44:193-9.

440. Ravazzoni, C., and R. Valerio. 1957. Action of lipotropic factors on the esterases of the liver. Boll. Chim. Farm. 96:239-43.

441. Raymond, M. J., and C. R. Treadwell. 1949. Lipotropic activity of various compounds under standardized conditions. Proc. Soc. Exptl. Biol. and Med. 70(1):43-45.

442. Reinhold, J. G. 1972. Nutritional osteomalacia in immigrants in an urban community. Lancet 1:386.

443. Rhodes, D. N., and C. H. Lea. 1958. On the composition of the phospholipids of cows' milk. J. Dairy Res. 25(1):60-69.

444. Ribereau-Gayon, J., and E. Peynaud. 1952. Use in wine industry of vitamins for promoting fermentation. Compt. Rend. Acad. Agr. France 39:444-8.

445. Ribereau-Gayon, J., E. Peynaud, and S. Lafourcade. 1955. Microbiological determination of meso-inositol with Kloeckera apiculata and Saccharomyces veronae applied to grapes and wine. Rev. Fermentations et Ind. Aliment. 10:119-21.

446. Ribereau-Gayon, P., and A. Bertrand. 1971. Simultaneous determination of organic acids, polyalcohols and sugars in wine. Applications. Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences, Serie D Science Naturelles 273(19):1761-1762.

447. Richardson, K. E. 1958. Metabolism of inositol. Univ. Microfilms LC Card No. Mic 58-3189, 75 pp; Dissertation Abstr. 19:948.

448. Richardson, K. E., and B. Axelrod. 1959. Conversion of inositol to carbon dioxide by rat-kidney preparations. Biochim. et Biophys. Acta 32:265-7.

449. Riddle, J. W. 1943. The effect of certain factoral deficiencies of the vitamin B complex on resistance to bacterial and viral infections in white mice. Ohio State Univ. Absts. Doctoral Diss. 43:143-150.

450. Ridgeway, G. J. 1954. A study of the function of inositol in yeast. Univ. Microfilms, Publ. No. 8105, 97 pp; Dissertation Abstr. 14:752-3.

* 451. Ridout, J. H., C. C. Lucas, J. M. Patterson, and C. H. Best. 1946. Lipotropic factors and the fatty liver produced by feeding cholesterol. Biochem. J. 40(4):494-499.

452. Ridout, J. H., C. C. Lucas, J. M. Patterson, and C. H. Best. 1954. Preventive and curative studies on the "cholesterol fatty liver" of rats. Biochem. J. 58(2):301-306.

453. Ridout, J. H., J. M. Patterson, C. C. Lucas, and C. H. Best. 1954. Effects of lipotropic substances on the cholesterol content of the serum of rats. Biochem. J. 58(2):306-312.

454. Ritchey, M. G., L. F. Wicks, and E. L. Tatum. 1947. Biotin, choline, inositol, p-aminobenzoic acid, and vitamin B6 in transplantable mouse carcinomas and in mouse blood. J. Biol. Chem. 171:51-9.

455. Ritschel, W. A., et al. 1970. Statistical evaluation of the effect of 3 stimulants of cellular metabolism. Praxis 59:1278-83.

456. Roberts, R. N. 1967. Gas chromatography of inositol and glycerol. Lipid Chromatogr. Anal. 1:446-63.

457. Romeo, D., et al. 1967. Protection effect of polyhydroxyl compounds on heart lysosomal structures. Experientia 23:247-8.

458. Rong, L. C. 1957. Effects of choline, p-aminobenzoic acid, and inositol on the growth of mice. Bitamin 13:508-13.

459. Roy, D. K. 1955. Effect of some salts and vitamins on the activity and stabilization of fungal alpha-amylase. Ann. Biochem. and Exptl. Med. 51(1):63-64.

460. Sachs, B. A., E. Danielson, B. Burack, and L. Leiter. 1958. In vitro and in vivo effects of inositol phosphatide (lipositol) fraction of soybean on serum lipides and lipoproteins. J. Appl. Physiol. 13:381-4.

461. Saruno, R., M. Matsuura, and K. Sasaki. 1955. The turbidity of synthetic sake. J. Soc. Brewing, Japan 50:463-6.

462. Sasaki, Y. 1916. Comparative studies of the behavior of the oxyhydroaromatic bodies toward animal oranisms. Act. Schol. Medic. Univ. Kioto 1:413-23.

463. Sato, K., K. Ueda, and T. Kurokawa. 1930. The fate of infused sugar with special consideration of the sugar-uptake capacity of tissue cells. VI. Behavior of lactacidogen, lactic acid and inositol in the muscles after sugar injection. Tohoku J. Exp. Med. 14:335-40.

464. Scevola, G. 1953a. Mesoinositol; biologic properties; clinical application. Minerva Med. 1:86-91.

465. Scevola, G. 1953b. Mesoinositol in prophylaxis of phleboyhrombosis; phlebographic histologic and experimental study. Minerva Med. 2:1635-1644.

466. Scevola, G. 1954. Antithromboplastic activity of meso-inositol and vitamin E. Boll. e Mem. Soc. Piemontese Chir. 24:159-64.

467. Scevola, G. 1954. Action of meso-inositol on coagulation. Proc. Intern. Conf. Thrombosis and Embolism. 1st, Basel 331-4.

468. Schaefer, A. E., W. D. Salmon, and O. M. Hale. 1950. The influence of riboflavin, pyridoxine, inositol, and protein-depletion-repletion upon the induction of neoplasms by choline deficiency. Cancer Research 10:786-92.

469. Scheunert, A., and H. Haenel. 1955. Liquid fruit and vegetable conserves as carriers of the vitamin B complex. Ind. Obst.- U. Gemueseverwert. 40:125-7.

470. Scheunert, A., and H. Haenel. 1956. The partition of the B-complex vitamins in the fruit juice and residue of fruits. Ernaehrungsforsch. 1:520-5.

471. Scheunert, A., and H. Haenel. 1956. Content of B-complex vitamins in some fruit mother liquors, their grounds, and the ground extracts prepared therefrom. Vitamine u. Hormone 7:310-15.

472. Schimassek, H., D. Kohl, and T. Buecher. 1959. Glycerylphosphorylcholine, the kidney substance, Ma-Mark, of Ullrich. Biochem. Z. 331:87-97.

473. Schneyder, J. 1965. Evolution of vitamins in grape juices. Bull. Office Intern. Vigne Vin 38(409):286-309.

474. Schopfer, W. H., T. Posternak, and M. L. Boss. 1948. Specificity of action of mesoinositol, essential growth factor for mutant of Neurospora crassa. Internat. Ztschr. Vitaminforsch. 20:121-128.

475. Schopfer, W. H. 1951. meso-Inositol in biology. Bull. Soc. Chim. Biol. 33:1113-46.

476. Schopfer, W. H., T. Posternak, and D. Wuestenfeld. 1960. Research on the functions and metabolism of meso-inositol. I. The morphogenetic effect of this factor upon a yeast, Schizosaccharomyces pombe. Arch. Sci. (Geneve) 13(4):522-530.

477. Schopfer, W. H., and T. Posternak. 1960. Research on the functions and metabolism of meso-inositol. III. Comparative physiological study of two anti-inositols, isomytilitol and the oxide of methylene-pentahydroxy-cyclohexane-1,3,5/4,6 acting on Schizosaccharomyces pombe. Arch. Sci. (Geneve) 13(4):537-541.

478. Schultz, A. S., and L. Atkin. 1947. The utility of bios response in yeast classification and nomenclature. Arch. Biochem. 14:369-80.

479. Scott, M. L. 1951. Enlarged-hock disorder in turkeys. II. Factors affecting the excretion and retention of creatine by young poults. Poultry Sci. 30:839-45.

480. Scriban, R. 1970. Vitamins of barley, malt and beer. A review. Annales de la Nutrition et de l'Alimentation 24(3):B377-99.

481. Sealock, R. R., and A. H. Livermore. 1943. The vitamin B complex of peeled wheat bread. J. Nutrition 25:265-74.

* 482. Sebrell, W. H., Jr., and R. S. Harris. 1954. The vitamins. Chemistry, physiology, pathology. Vol. II, xii + 766 p. Academic Press Inc.: New York 10.

483. Sechi, A. M. 1958. The vitamin contents in a variety of Italian rice and its byproducts. J. Vitaminol. 4(2):114-117.

484. Seckfort, H. 1954. Metabolic studies. Arztl. Forsch. 8:II/101-II/102; II/118-II/121.

485. Selik, M., and H. Ziegler. 1969. Sugar, protein and vitamin contents of berry juice of Juniperus drupacea Labill. Qualitas Plantarum et Materiae Vegetabiles 17(4):265-72.

486. Sellers, E. A. 1946. Effect of lipotropes on experimental cirrhosis. Conf. on Liver Injury, Trans. 5:16-21.

487. Sequeira, R. M., and R. B. Lew. 1970. The carbohydrate composition of almond hulls. J. Agricultural and Food Chemistry 18(5):950-51.

488. Setchell, B. P., R. M. C. Dawson, and R. W. White. 1968. The high concentration of free myo-inositol in rete testis fluid from rams. J. Reprod. Fert. J. Reprod. Fert. 17(1):219-20.

489. Sherman, W. R., et al. 1968. The identification of myo-inosose-2 and scyllo-inositol in mammalian tissues. Biochemistry (Wash.) 7:819-24.

490. Sherman, W. R., et al. 1968. The measurement of myo-inositol, myo-inosose-2 and scyllo-inositol in mammalian tissues. Biochim. Biophys. Acta 158:197-205.

491. Shimizu, T. 1921. Chemical composition of the brain. Biochm. Ztschr. 117:252-62.

492. Sieburth, J. F., and B. A. McLaren. 1953. Growth studies with the cockroach, Periplaneta americana, fed vitamin-deficient diets substituted with corresponding antivitamins. Ann. Entomol. Soc. Am. 46:43-8.

493. Simpson, J. S., and D. F. Harris. 1957. Experience of the microbiological assay of vitamins and amino acids by large-plate methods. Analyst 82:210-11.

494. Smith, M. I., E. W. Emmart, and W. T. McClosky. Streptomycin in experimental guinea pig tuberculosis. Am. Rev. Tuberculosis 58(1):112-122.

495. Snell, E. E. 1948. Use of microorganisms for assay of vitamins. Physiol. Rev. 28(2):255-282.

496. Snell, E. E., and L. D. Wright. 1950. The water-soluble vitamins. Ann. Rev. Biochem. 19:277-318.

497. Soederhjelm, L., and B. Zetterberg. 1948. Inhibition of the bacteriostatic activity of streptomycin and gamma-hexachlorocyclohexane by inositol-containing substances. Upsala Lakareforen. Forh. 53:235-40; Abstracts World Med. 6:118-19.

* 498. Sonne, S., and H. Sobbotka. 1947. Inositol content of blood plasma. Arch. Biochem. 14:93-6.

499. Spitzer, R. R., and P. H. Phillips. 1946. Alopecia in rats fed certain soybean-oil meal rations. Proc. Soc. Exptl. Biol. Med. 63:10-13.

500. Springer, G. F., et al. 1966. Free myo-inositol as major component of Paeonia lactiflora syrup. Naturwissenschaften 53:277.

501. Stamler, J., C. Bolene, R. Harris, and L. N. Katz. 1950a. Effect of choline and inositol on plasma and tissue lipids and atherosclerosis in cholesterol-fed chick. Circulation 2:714-721.

502. Stamler, J., C. Bolene, R. Harris, and L. N. Katz. 1950b. Effect of choline and inositol on plasma and tissue-lipides and atherosclerosis in the cholesterol-fed chick. Circulation 2:714-21.

503. Stamler, J., C. Bolene, R. Harris, and L. N. Katz. 1950c. Effect of choline and inositol on plasma and tissue lipids and on spontaneous and stilbestrol (diethyl-stilbestrol, estrogen)-induced atherosclerosis in chick. Circulation 2:722-725.

* 504. Staubach, H., and H. Seckfort. 1954. Inositol and blood picture. Klin. Wochschr. 32:567.

505. Steinkraus, K. H., and R. A. Morse. 1966. Factors influencing the fermentation of honey in mead production. J. Apicult. Res. 5(1):17-26.

* 506. Stetten, M. R., and D. W. Stetten, Jr. 1946. Biological conversion of inositol into glucose. J. Biol. Chem. 164:85-91.

507. Stewart, M. A., M. M. Kurien, W. R. Sherman, and E. V. Cotlier. 1968. Inositol changes in nerve and lens of galactose fed rats. J. Neurochem. 15(9):941-946.

508. Storck, R. 1947. Growth factors of yeast. Rev. Fermentations et Inds. Aliment. 2:8-23.

509. Strickland, K. P., W. Thompson, D. Subrahmanyam, and R. J. Rossiter. 1960. Biosynthesis of brain inositol phosphatide. Biochem. J. 76:41p-42p.

510. Strickland, R. D., P. A. Mack, F. T. Gurule, T. R. Podleski, O. Salome, and W. A. Childs. 1959. Determining serum proteins gravimetrically after agar electrophoresis. Anal. Chem. 31:1410-13.

511. Studer, P., and P. Haefelfinger. 1968. Thin-layer chromatographic determination of meso-inositol on precoated plates. J. Chromatogr. 33(2):385-90.

512. Sullivan, B. J., and P. M. Flynn. 1957. Influence of inositol on peripheral vascular response to cold injury. Proc. Soc. Exptl. Biol. Med. 94:346-7.

513. Sullivan, B. J., and M. F. LeBlanc. 1957. Effect of inositol and rapid rewarming on extent of tissue damage due to cold injury. Am. J. Physiol. 189:501-3.

514. Sure, B. 1941. Dietary requirements for fertility and lactation; role of inositol; preliminary report. Science 94:167.

* 515. Sure, B. 1943. Dietary requirements for fertility and lactation. XXXI. Further studies on the role of p-aminobenzoic acid and inositol in lactation and growth of the albino rat. J. Nutrition 26(3):275-283.

516. Sure, B., and F. Romans. 1948. Influence of the concentration of mixtures of various components of the vitamin B complex on biological value of casein and on economy of food utilization. J. Nutrition 36:727-37.

517. Suzuki, Y. 1962. Biochemistry of inositol. Maikurobaioassei 4:1-11.

518. Synder, F., W. E. Cornatzer, and G. E. Simonson. 1957. Comparative lipotropic and lipide phosphorylating effects of choline, betaine, and inositol. Proc. Soc. Exptl. Biol. and Med. 96(3):670-672.

519. Szymanowski, W., B. Wisniewski, B. Golebiowski, and L. Olszewska. 1958. Application of pressed-powder methods to investigations of infrared absorption spectra of solids. Bull. Acad. Polon. Sci., Ser. Sci. Math., Astron. et Phys. 6:223-31.

520. Szymanowska, Z. 1962. Infrared spectrophotometric determination of m-inositol. Acta Physiol. Polon. 13(1):205-215.

521. Takahashi, T., K. Kimoto, and S. Minami. 1952. Technical organic analysis by ceric sulfate titration. I. Volumetric determination of polyhydric alcohols. J. Chem. Soc. Japan. Ind. Chem. Sect. 55:115-16.

522. Takaku, I., K. Hasegawa, R. Kimura, and Y. Watanabe. 1967a. Biochemical characteristics of the cataract lens. I. Comparison of water content and concentration of sorbitol, inositol, glutathione, and sialic acid. Nippon Ganka Gakkai Zasshi (Acta Soc. Ophthalmol. Jap.) 71(7):305-9.

523. Takaku, I., et al. 1967b. Biochemical characteristics of the cataract lens. 1. Comparison of water content and concentration of sorbitol, inositol, glutathione and sialic acid in the young and adult rat lens. Acta Soc. Ophthal. Jap. 71:1029-33.

524. Tani, Y., H. Tani, and Y. Tani. 1956. Preparation of sake mash from purified starch. Japan. Pat. 4988.

525. Tani, Y. 1956. Content of vitamin B group in sake on the market. J. Fermentation Technol. (Japan) 34:428-31.

526. Tarantola, C., and R. Lovisolo. 1955. Evolution of nitrogenous substances during the manufacture of Asti spumante from Canelli muscat grapes, and the influence of certain vitamins upon the refermentation. Ann. Sper. Agrar. (Rome) 9:167-88.

527. Tatum, E. L., M. G. Ritchey, E. V. Cowdry, and L. F. Wicks. 1946. Vitamin content of mouse epidermis during methyl-cholanthrene carcinogenesis. I. Biotin, choline, inositol, p-aminobenzoic acid, and pyridoxine. J. Biol. Chem. 163:675-82.

528. Taylor, W. E., and J. M. McKibbin. 1952. Effect of choline on yeast bioassay of inositol. Proc. Soc. Exptl. Biol. and Med. 79(1):95-96.

529. Taylor, W. E., and J. M. McKibbin. 1953. Determination of lipid inositol in animal tissues. J. Biol. Chem. 201:609-613.

530. Tazaka, S., H. Naito, T. Tomono, K. Naito, T. Kumagai, M. Usui, and K. Kobayakawa. 1957. Experimental atherosclerosis. The effects of various pharmaceuticals on aortic atherosclerosis in cholesterol-fed chickens. Sogo Igaku 14:45-7.

531. Teramoto, S., and W. Hashida. 1953. The nutrient substances of hiochi-bacteria (putrefactive bacteria of sake) and their antagonists. J. Fermentation Technol. (Japan) 31:334-8.

532. Torda, C., and H. G. Wolff. 1946. Effect of some isocyclic, aromatic, and heterocyclic compounds on muscle sensitivity to acetylcholine and potassium. Am. J. Physiol. 145:608-14.

533. Totter, J. R. 1957. Water-soluble vitamins. I. Vitamin B12, folic acid, choline, and p-aminobenzoic acid. Ann. Rev. Biochem. 26:181-208.

534. Tsuyuki, H., and D. R. Idler. 1960. The metabolism of inositol in salmon. I. The distribution of radioactivity in acid-soluble extracts of coho salmon following intraperitoneal and intra-arterial injections of myoinositol-2-C14. Can. J. Biochem. and Physiol. 38:1173-6.

535. Tsuyuki, H., and D. R. Idler. 1961. The metabolism of inositol in salmon. III. The biochemical reactions of 2-C14-myoinositol in coho liver. Canadian J. Biochem. and Physiol. 39(6):1037-1042.

536. Ueno, T., N. Kurihara, and M. Nakajima. 1967. Studies of synthesis of carbohydrate antibiotics. III. Gas-liquid chromatography of trifluoroacetyl derivatives of carbohydrates including cyclitols. Agr. Biol. Chem. (Tokyo) 31 (10):1189-94.

537. Vanderzant, E. S. 1959. Inositol: An indispensable dietary requirement for the boll weevil. J. Econ. Ent. 52(5):1018-1019.

538. Vanderzant, E. S., and C. D. Richardson. 1964. Nutrition of the adult boll weevil. Lipid requirements. J. Insect Physiol. 10(2):267-72.

539. Vanek, Z. 1958. Substances stimulating the production of chlortetracycline by a low-production strain of Streptomyces aureofaciens. Folia Biol. (Prague) 4(2):100-106.

540. Verona, O. 1951. Auxological research on yeasts, especially of wine fermentation. Ann. Univ. Pisa, Facolta Agrar. 12:105-22.

541. Vogel, W. H., R. Snyder, and M. P. Schulman. 1964. Effects of aromatic and nonaromatic model compounds and drugs on enzymic activities. J. Pharmacol. Exptl. Therap. 146(1):66-73.

542. Voit, K., and H. Seckfort. 1953. Metabolic effect. Muenchen. Med. Wchnschr. 95:680-681.

543. Volk, W. A., and D. Pennington. 1951. The fermentation of inositol. J. Bact. 61:469-73.

* 544. Vorhaus, M. G., M. D. Michael, L. Gompertz, and A. Feder. 1943. Clinical Experiments with riboflavin, inositol and calcium pantothenate. Am. J. Digest. Diseases 10:45-48.

545. Vuillaume, R. 1948. Meso-inositol, a vitamin of group B. Rec. Med. Vet. (Ecole d'Alfort) 124(10):433-439.

546. Wagenknecht, A. C., L. M. Lewin, and H. E. Carter. 1959. Phosphatidyl inositol of peas. J. Biol. Chem. 234:2265-8.

547. Wainer, A. 1967. The chromatography of ninhydrin negative compounds on an amino acid analyzer column. J. Chromatogr. 26:48-53.

548. Walenrod, L., et al. 1971. Elimination of choline interference in microbiological assay of inositol in pharmaceutical products. J. Pharm. Sci. 60:1856-7.

549. Walker, H. G., Jr. 1965. GLC (gas-liquid chromatography) examination of molasses carbohydrates. Intern. Sugar J. 67(800):237-9.

550. Walton, R. P., R. P. Walton, Jr., and W. L. Thompson. 1959. Inotropic activity of catechol isomers and a series of related compounds. J. Pharmacol. and Exptl. Therap. 125(3):202-207.

551. Webb, A. M., and J. R. Loofbourow. 1947. Intercellular hormones. VI. Release and synthesis of factors of the vitamin B complex by damaged living cells. Biochem. J. 41:114-19.

552. Webb, S. J. 1959. Chloramphenicol and the survival of airborne bacteria. Nature 183:1072.

553. Webb, S. J., C. A. M. Cook, and R. Bather. 1964. Inositol and the protection of cells. I. The release of nucleic acids and viruses from bacterial and mammalian cells. Canadian J. Biochem. 42(2): 157-166.

554. Webb, S. J., and M. D. Dumasia. 1967. Bound water, inositol, and the induction of lambda prophages by ultraviolet light. Can. J. Microbiol. 13(3):303-12.

555. Webb, S. J. 1967. The influece of oxygen and inositol on the survival of semidried microorganisms (Escherichia coli, Bacillus subtilis, Serratia marcocens, isotope study, molecular biology. Can. J. Microbiol. 13(7):733-742.

* 556. Weidlein, E. R., Jr. 1951. The biochemistry of inositol. Mellon Inst. Bibliograph Ser. Bull. 6:1-53.

557. Weinfurtner, F., F. Eschenbecher, and K. H. Hettmann. 1966. Meso-inositol in beer fermentation. Brauwissenschaft 19(5): 174-7.

558. Weiss, S. B., L. Marx, and W. Marx. 1952. The effects of thyroid and of choline and inositol on cholesterol distribution in rats fed a high cholesterol diet. Endocrinology 50(2):192-198.

559. Weissbach, A. 1958. The enzymic determination of myo-inositol. Biochim. et Biophys. Acta 27(3):608-611.

560. Wells, W. W., J. P. McIntyre, D. J. Schlichter, M. Wacholtz, and S. E. Spieker. 1969. Myoinositol metabolism in galactosemia. Ann. N.Y. Acad. Sci. 165:599-608.

561. Wells, H. J., and W. W. Wells. 1967. Galactose toxicity and myoinositol metabolism in the developing rat brain. Biochemistry 6(4):1168-1173.

562. Wells, I. C., et al. 1968. Effects of dietary deficiencies of lipotropic factors on plasma cholesterol esterification and tissue cholesterol in rats. J. Nutr. 95:55-62.

563. Whitaker, J. R., A. L. Tappel, and E. Wormser. 1962. Modification of enzymic activity. I. Effect of organic additives on alpha-amylase activity. Biochim. Biophys. Acta 62:300-9.

564. White, I. G. 1954. The effect of some seminal constituents and related substances on diluted mammalian spermatozoa. Australian J. Biol. Sci. 7(3):379-390.

565. White, J., and D. J. Munns. 1950. Yeast growth method for determination of biotin, pantothenic acid, and inositol activities in raw materials. J. Inst. Brewing 56:141-9.

566. White, J., and D. J. Munns. 1953. Quantitative studies on the importance of nutrilites in yeast production. Am. Brewer 86(5): 29-32.

* 567. Wiebelhaus, V. D., J. J. Bethell, and H. A. Lardy. 1947. The metabolism of meso-inositol in the rat. Arch. Biochem. 13:379-388.

568. Wierzuchowski, M., and F. Sekuracki. 1935. Cleavage, oxidation, and energy conversion in the dog. 2. Organic source and orifices of lactic acid during conversion of fructose, on the basis of control experiments with inositol, acetaldehyde and sodium lactate. Biochem. Z. 276:112-31.

569. Wiken, T., and O. Richard. 1951. Investigations of the physiology of wine yeasts. I. The conditions of growth of an auxo-autotrophic Swiss culture yeast. Antonie van Leeuwenhoek, J. Microbiol. & Serol. 17(4):209-226.

570. Wiken, T., and O. Richard. 1952. The physiology of wine yeasts. III. Growth requirement of an auxoheterotrophic Swiss culture wine yeast. Antonie van Leewenhoek J. Microbiol. Serol. 18:293-315.

571. Wiken, T., and C. Agthe. 1958. Function of the nutrients meso-inositol, (+)-biotin, D(+)-pantothenic acid, beta-alanine, and D(-)-pantoic acid for the growth of the wine yeast Dezaley (Saccharomyces cerevisiae). Arch. Mikrobiol. 31:289-304.

572. Wilkinson, C. F., Jr. 1955. Effects of lipotropes and sitosterol on the level of blood lipids and the clinical course of angina pectoris. J. Am. Geriat. Soc. 3(6):381-388.

573. Williams, H. L., and E. M. Watson. 1947. The effects of various compounds upon the transamination enzyme activity of rat kidney tissue. Rev. Canadienne Biol. 6(1):43-52.

* 574. Williams, R. J. 1942a. The approximate vitamin requirements of human beings. J. Am. Med. Assn. 119(1):1-3.

575. Williams, R. J. 1942b. Microbiological assay methods. Univ. Texas Pub. No. 4237:7-13.

576. Williams, R. J., and D. H. Saunders. 1934. The effect of inositol, crystallized vitamin B1 and "Pantothen" acid on the growth of various yeast cultures. Biochemical J. 28:1887-93.

577. Winter, L. B. 1934. Inositol metabolism in the mammal heart. Biochemical J. 28:6-10.

578. Wiss, O. 1950. Microbiological determinations of vitamins and amino acids (Suggestions of the Swiss Food Research Commission). Mitt. Lebensm. Hyg. 41:225-58.

579. Wolf, L. E. 1951. Diet experiments with trout. Progressive Fish Culturist 13(1):17-24.

580. Woolley, D. W. 1941a. Relationship of pantothenic acid and inositol to alopecia in mice. Proc. Soc. Exper. Biol. & Med. 46:565-569.

581. Woolley, D. W. 1941b. Identification of the mouse antialopecia factor. J. Biol. Chem. 139(1):29-34.

582. Woolley, D. W. 1941c. A method for the estimation of inositol. J. Biol. Chem. 140(2):453-459.

583. Woolley, D. W. 1941d. Biologic Specificity. J. Biol. Chem. 140:461-466.

584. Woolley, D. W. 1942a. Changes in the form of inositol during incubation of eggs. Proc. Soc. Exp. Biol. and Med. 49(4):540-541.

585. Woolley, D. W. 1942b. Bacterial synthesis of inositol and its importance in the nutritional economy in mice. J. Bact. 43(1):5.

586. Woolley, D. W. 1944. The nutritional significance of inositol. J. Nutrition 28(5):305-314.

587. Woolley, D. W. 1947a. Water-soluble vitamins. Ann. Rev. Biochem. 16:359-386.

588. Woolley, D. W. 1947b. The determination of inositol. In: Biological Symposia, edited by Jaques Cattell, et al. Vol. XII. pp279-289.

589. Yagi, K., A. Kotaki, and Y. Yamamoto. 1965. Studies on myo-inositol: I. Effect on the growth of rats. J. Vitaminol. 11(1):14-19.

590. Young, L. 1934a. Volumetric determination of inositol. Biochem. J. 28:1428-1434.

591. Young, L. 1934b. Determination of inositol in animal tissue. Biochem. J. 28:1435-1443.

592. Zechmeister, L. and P. Szecsi. 1921. The occurrence of fumaric acid and of inositol. Ber. Dtsch. Chem. Ges. 54:172-173.

593. Zubkova, R. D. 1949. Vitamin characterization of five yeast strains. Izvest. Akad. Nauk Kazakh. SSR., Ser. Mikrobiol. 1(62):61-65.

594. Anon. 1960. Effects of dietary fat, inositol, vitamin B12 and glucose cyclo-acetoacetate on coagulation. Nutr. Rev. 18:246-247.

595. Anon. 1942. Inositol, anti-alopecia factor. Am. Prof. Pharmacist 8(8):502, 536.

596. Anon. 1953. Inositol literature briefs. Corn Products Refining Co., Tech. Bull. Y3-101, 44pp.

Ann. Internal. Med. 20:580-589; 1944

METABOLIC STUDIES IN PATIENTS WITH CANCER OF THE GASTROINTESTINAL TRACT. IX. EFFECTS OF DIETARY CONSTITUENTS UPON THE CHEMICAL COMPOSITION OF THE LIVER, ESPECIALLY IN PATIENTS WITH GASTROINTESTINAL CANCER*

By JULES C. ABELS, M.D.,† IRVING M. ARIEL, M.D.,‡ HELEN T. MURPHY, PH.D., GEORGE T. PACK, M.D., and C. P. RHOADS, M.D., F.A.C.P.,
New York, N. Y.

INTRODUCTION

In a previous investigation the livers of patients with gastrointestinal cancer were found to have abnormally high concentrations of fat and probably low concentrations of glycogen.[1] This may be of clinical significance because, from the evidence, these changes render the liver abnormally susceptible to injury.[2] The physiological effects of these two constituents may be interrelated, as suggested by the fact that the livers of individuals exposed to several hepatotoxins are not damaged when considerable amounts of glucose are administered.[3] This effect apparently is not due simply to the increased amounts of hepatic glycogen deposited, but to the fact that when adequate stores of carbohydrate are available for metabolic purposes, fatty infiltration of the liver is prevented.[4]

The abnormal hepatic chemical composition is particularly important in patients with gastrointestinal cancer because they frequently are subjected to considerable anesthesia, medication, and surgical manipulation. Since in normal individuals these procedures induce hepatic damage,[5, 6] it is reasonable to assume that the damage is considerably greater in a liver abnormally susceptible to injury because of its altered constitution.

In experimental animals, fatty infiltration of the liver can be reduced by the administration of certain dietary constituents. Moreover, the same measures apparently increase the functional capacity of the organ and its resistance to the usual injurious effects of several noxious agents.[7, 8] A clinical study, therefore, was undertaken to ascertain whether or not the preoperative administration of the same dietary factors to the patients with gastrointestinal cancer would restore the chemical composition of their livers to normal. The results of that study form the subject of the present report.

* Received for publication June 9, 1943.
From The Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York City.
The authors gratefully acknowledge a grant from the National Cancer Institute.
† Finney-Howell Fellow.
‡ Trainee in diagnosis and treatment, National Cancer Research Institute.

Material

Thirty-seven patients with gastrointestinal cancer were studied. Of these, three had carcinoma of the terminal esophagus, 29 of the stomach, and five of the colon. As controls, 12 patients with benign gastrointestinal lesions were used. Of these, five had gastric ulcer, two atrophic gastritis, two gastric polyposis, one chronic ulceration of the terminal esophagus, and two ulcers of the duodenum. As a second control group, eight patients with obstructive lesions of the common bile duct were studied. In three this obstruction was due to carcinoma of the head of the pancreas, in four to impacted gall stones, and in one to a stenosis of the ampulla of Vater.

The final diagnosis in all patients was made at laparotomy and by microscopic examination of the biopsied lesion.

Methods

The preoperative care, dietary régime, and anesthesia of all patients, as well as the technics by which the liver tissues were obtained and analyzed have been reported previously.[1]

At 10 p.m. of the night before the day of operation, a small Levin tube was inserted into the stomach of each patient. This tube was allowed to remain in place until 7 a.m., and through it were introduced, every two hours for five doses, the test materials studied. At 7 a.m., one hour after the last tube feeding, the stomach contents were aspirated and discarded.

The glucose was administered in the form of 200 ml. of 25 per cent solution, so that in the five doses 250 gm. were given. The lipocaic employed was made according to the directions of Dragstedt,* and introduced through the tube in 1.6 gm. amounts suspended in 100 ml. of water. A 10 per cent aqueous solution of choline chloride was administered in five doses each of 0.6 gm. Inositol † was given in 240 mg. amounts five times, total 1,200 mg.

Results

The results are presented in four parts as the effects of the preoperative administration of (*a*) glucose, (*b*) lipocaic, (*c*) choline chloride, and (*d*) inositol on the chemical constitution of the livers studied.

A. Glucose: For a group of 18 fasted patients with gastrointestinal cancer, the hepatic glycogen previously was found to average 2.91 gm. per cent,[1] the fat 17.47 gm. per cent, and the protein 16.17 gm. per cent (table 1).

In contrast to these values were those of the livers of a group of 11 patients with gastrointestinal cancer who received preoperatively 250 gm. of glucose (table 2). In these, glycogen varied from 2.3 to 9.95 gm. and averaged 4.39 gm. per cent or 1.4 times that of the fasted control patients. The fat ranged from 3.9 to 15.2 gm. and averaged only 7.24 gm. per cent, or

* Procured through the courtesy of Eli Lilly & Co., Indianapolis, Ind.
† Procured through the courtesy of Lederle & Co., Pearl River, N. Y.

TABLE I

The Chemical Constitution of the Livers of Fasted Patients

Number of cases	Disease	Concentrations of Hepatic					
		Glycogen, gm. per cent		Fat, gm. per cent		Protein, gm. per cent	
		Range	Average	Range	Average	Range	Average
18	Gastrointestinal carcinoma	1.0–7.3	2.91	5.3–35.0	17.47	12.9–17.95	16.17
4	Bile duct obstruction	1.3–5.1	2.62	11.5–26.6	17.72	12.3, 14.9	
4	Benign disorders of gastrointestinal tract	1.3–3.55	2.28	6.2–17.0	11.8	11.4–16.6	14.6

TABLE II

The Chemical Composition of the Livers of Patients with Gastrointestinal Cancer Who Received Preoperatively 250 gm. of Glucose

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent	Total Protein, gm. per cent
M. K.	Normal	4.2	8.1	13.8
E. V.	Normal	9.95	6.1	11.9
L. G.	Normal	3.1	15.2	17.9
T. C.	Normal	4.7	5.7	14.2
C. C.	Normal	3.0	5.4	15.9
A. A.	Normal	3.2	7.3	15.8
S. W.	Normal	4.5	4.55	16.3
O. B.	Normal	2.5	3.9	15.1
W. G.	Normal	5.2	4.7	
[illegible]	Normal	5.6	10.3	17.0
[illegible]	Normal	2.3	10.3	
	Average.....	4.39	7.24	15.32

0.42 that of the fasted control group. Of the 10 patients fed glucose, three [illegible] abnormally high concentrations of liver fat (greater than 8.5 gm. per cent).

[illegible] of the 11 instances in which the protein contents of the livers were [illegible] were found to range from 13.8 to 17.9 gm. and average 15.[illegible] or about equal to that of the group of patients who received no glucose.

The preoperative ingestion of glucose apparently affected the chemical constitution also of the livers of patients with obstructive bile duct disease. In the preceding study the hepatic glycogen concentrations of four individuals with this disorder who were fasted before operation, averaged 2.62 gm. per cent, the fat averaged 17.72 gm. per cent, and the protein 13.5 gm. per cent (Table I).

Four individuals with common bile duct obstruction were given preoperatively 250 gm. of glucose. The glycogen values varied from 2.4 to 4.6 gm. and averaged 3.05 gm. per cent, or 1.2 times that of the fasted controls.

The concentrations of fat in their livers were found to range from 3.8 to 8.85 gm. and average 6.40 gm. per cent, or 0.31 that of the fasted group. No significant difference was found between the contents of protein in the livers of the fasted and of the patients treated with glucose (table 3).

TABLE III

The Chemical Composition of the Livers of Patients with Bile Duct Obstruction Who Received Preoperatively 250 gm. of Glucose

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent	Total Protein, gm. per cent
L. H.	Bile stasis, periductal fibrosis	2.8	6.95	12.2
L. L.	Atrophy, central pigmentation	4.6	8.85	14.5
L. I.	Bile stasis	2.4	6.0	12.5
M. W.	Atrophy, bile stasis	2.4	3.8	13.0
	Average..	3.05	6.40	13.00

Similar studies were made in a group of patients with benign gastrointestinal disorders. In the livers of four fasted patients previously reported the glycogen averaged 2.28 per cent, the fat 11.8 gm. per cent, and the protein 14.6 gm. per cent (table 1).

In contrast, in a group of six patients with benign disorders who were fed preoperatively 250 gm. of glucose, the hepatic concentration of glycogen was found to be considerably increased. These values ranged from 5.25 to 9.45 gm. and averaged 7.35 gm. per cent, or 3.4 times that of the control group (table 4). It is interesting to note that the average increase of glycogen in the livers of patients with benign gastrointestinal lesions who received glucose preoperatively is considerably greater than that of patients with gastrointestinal cancer or obstructive biliary disease who received the same amount of carbohydrate.

TABLE IV

The Chemical Composition of the Livers of Patients with Benign Gastrointestinal Disorders Who Received Preoperatively 250 gm. of Glucose

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent	Total Protein, gm. per cent
F. H.	Normal	6.2	2.3	14.3
J. S.	Normal	7.0	6.35	11.4
A. H.	Normal	9.45	6.2	
J. H.	Normal	7.0	7.85	15.8
D. E.	Normal	9.2	8.5	
A. U.	Normal	5.25	5.95	
	Average..	7.35	6.19	13.80

The fat content of the livers of the six glucose-treated patients with benign disorders ranged from 2.3 to 8.5 gm. and averaged 6.19 gm. per cent, or 0.53 times that of the control group. The protein concentrations ranged from 11.4 to 15.8 gm. and averaged 13.80 gm. per cent (table 4), a value equal to that of the fasted group.

Hence, the preoperative ingestion of glucose by the three groups of patients studied decreased the content of fat in their livers. The stores of hepatic glycogen were markedly increased only in the patients with benign gastrointestinal disease, a fact which suggests that hepatic glycogenesis or glycogen storage in patients with gastrointestinal cancer is impaired. These individuals are known to have hepatic dysfunction but no morphologic liver damage.[1] Extensive impairment of glycogenesis or glycogen storage apparently exists, too, in the patients with common bile duct obstructions who are known to have both functional and anatomic damage. Finally, the ingestion of glucose was without significant effect on the protein concentration in the livers of the three groups of patients studied.

TABLE V

The Chemical Composition of the Livers of Patients with Gastrointestinal Cancer Who Received Preoperatively 8 gm. of Lipocaic

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent	Total Protein, gm. per cent
H. D.	Normal	1.6	9.3	
A. McH.	Normal	4.3	7.45	
H. E.	Normal	1.6	6.9	
A. v. V.	Normal	3.6	7.2	
H. M.	Normal	2.4	9.35	
F. S.	Normal	2.25	11.1	
S. H.	Normal	2.9	7.35	15.2
S. E.	Normal	2.9	7.0	18.7
A. E.	Normal	1.8	7.8	14.5
[illegible] H.	Normal	2.5	4.05	
[illegible] U.	Normal	3.5	11.1	
	Average..	2.67	8.05	16.13

B. Lipocaic: The administration of lipocaic to animals with fatty infiltration of the liver effectively reduces their abnormal concentrations of hepatic lipid.[10] Whether the lipotropic effect of this material is due to its content of choline and inositol * or to some other factor still is unknown. The effects of this lipotropic material on the fat content of *human* livers thus far have not been ascertained. Were it possible by this means to decrease the excess fat content in the livers of patients subjected to hepatotoxins or to surgical operations, a valuable therapeutic agent might be at hand for the prevention of liver damage.

Eight grams of lipocaic divided into five equal doses were administered to a group of 11 patients with gastrointestinal cancer during their last 10 preoperative hours. The concentrations of fat in the livers of these individuals were found to range from 4.05 to 11.1 gm. and average 8.05 gm. per cent, or only 0.46 that of the group of 18 fasted patients with the same disorders. Of the 11 patients who received lipocaic, only four had slightly

* The choline and inositol contents of the lipocaic used were found to be 2.6 and 3.5 per cent respectively. These determinations were made for us by Dr. W. Wooley of the Rockefeller Institution of Medical Research and by the Laboratories of the Standard Brands, Inc.

abnormal concentrations of liver fat. The administration of lipocaic apparently had no significant effect on the concentrations of hepatic glycogen in these individuals, but, of course, no such effect was expected (table 5).

The concentrations of lipid in the livers of two patients with benign gastrointestinal lesions who each received 8 gm. of lipocaic preoperatively were 7.9 and 9.8 gm. per cent (table 6). Four patients with obstruction of the common bile duct were treated similarly and only two had normal hepatic concentrations of lipid. These concentrations in the four patients were 7.85, 8.4, 12.7, and 19.5 gm.

TABLE VI

The Chemical Composition of the Livers of Patients with Benign Gastrointestinal Disorders Who Received Preoperatively 8 gm. of Lipocaic

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent
F. N.	Normal	1.5	9.8
H. S.	Normal	3.0	7.9

It appears, then, that the administration of lipocaic to patients with gastrointestinal cancer subjected to operation significantly reduces the abnormally high content of fat in their livers. This did not uniformly occur in patients with obstruction of the bile ducts.

C. Choline Chloride: When it was apparent that the administration of lipocaic to patients with cancer of the gastrointestinal tract could reduce the concentrations of fat in their livers, it became of interest to ascertain whether or not this effect was due to the choline or inositol contents of the material. Both of these compounds are known to have a lipotropic effect in experimental animals.[11, 12]

TABLE VII

The Chemical Composition of the Livers of Patients with Bile Duct Obstruction Who Received Preoperatively 8 gm. of Lipocaic

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent
F. B.	Atrophy, bile stasis	3.3	12.7
L. S.	Bile stasis, periductal fibrosis	2.5	19.5
G. W.	Bile stasis, periductal fibrosis	2.8	8.4
E. T.	Bile stasis	1.1	7.85

Hence, seven patients with gastrointestinal cancer each were given a total of 3 gm. of choline chloride, or about 12 times the amount in the lipocaic administered. The concentrations of fat in the livers of these seven patients were found to range from 6.9 to 16.1 gm. and average 11.21 gm. per cent, or 0.64 that of the fasted patients (table 8). Of these seven values, four were above the upper limit of normal. Therefore, the lipotropic effect

of lipocaic was significantly greater than that of comparatively large doses of choline, and could not be attributed entirely to its choline content.

The administration of choline had no apparent effect on the hepatic concentration of glycogen.

TABLE VIII

The Chemical Composition of the Livers of Patients with Gastrointestinal Cancer Who Received Preoperatively 3 gm. of Choline Chloride

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent
P. R.	Normal	1.1	6.9
F. G.	Normal	2.3	11.0
F. L.	Normal	2.6	16.1
A. R.	Normal	2.7	8.2
H. B.	Normal	2.3	16.0
M. G.	Normal	4.74	8.3
T. S.	Normal	6.6	12.0
	Average..	3.19	11.21

D. Inositol: Inositol was given in five equal doses of 240 mg. to eight patients with gastrointestinal cancer. The total amount ingested by each was equal to four times that contained in the lipocaic administered. At the time of laparotomy the average hepatic concentration of glycogen was 3.28 gm. per cent. That of fat was 6.94 gm. per cent, or 0.39 that of the controls. In only one instance was the fat content abnormally elevated (table 9).

TABLE IX

The Chemical Composition of the Livers of Patients with Gastrointestinal Cancer Who Received Preoperatively 1.2 gm. of Inositol

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent
S. S.	Normal	4.95	4.55
M. M. N.	Normal	3.0	6.7
E. E.	Normal	2.1	7.05
A. H.	Normal	4.25	16.7
A. W.	Normal	1.5	5.7
E. G.	Normal	3.2	5.6
J. L.	Normal	3.6	5.7
J. K.	Normal	3.6	3.5
	Average..	3.28	6.94

TABLE X

The Chemical Composition of the Livers of Patients with Benign Gastrointestinal Disorders Who Received Preoperatively 1.2 gm. of Inositol

Patient	Histologic Appearance of Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent
F. S.	Normal	3.6	7.5
W. O.	Normal	2.9	7.0
S. S.	Normal	2.6	5.1
M. A.	Normal	3.2	4.9
	Average...	3.08	8.12

The same amounts of inositol also were administered preoperatively to four patients with gastric ulcer. The average glycogen content of their livers was found to be 3.08 gm. per cent and of lipid 8.12 gm. per cent (table 10).

The significantly decreased concentration of fat in the livers of the patients fed inositol suggests that the lipotropic effect of lipocaic may be due to its content of inositol. Experiments now are under way to ascertain the effect on the hepatic lipid of inositol in amounts just equal to that contained in the lipocaic administered, both when given alone and with choline.

Discussion

Previous studies have indicated that excessive lipid deposition in the livers of experimental animals can be prevented by the administration of carbohydrate [4] and of certain lipotropic factors.[10] It was reasonable to assume, therefore, that these measures also could reduce the lipid infiltration in the livers of human beings. This assumption now has been borne out by the data presented. The preoperative administration of glucose was found to exert a significant lipotropic effect on the liver fat of all patients studied, even though it did not increase significantly the hepatic glycogen stores in the patients with gastrointestinal tract cancer or with common bile duct obstruction. However, there is considerable evidence that the mere presence of hepatic glycogen stores does not necessarily afford protection to the liver nor is it always associated with a decreased content of hepatic lipid. The liver with a large store of glycogen but infiltrated with fat ordinarily is readily susceptible to damage, whereas the liver with depleted glycogen stores but with a low lipid content withstands the deleterious effect of many hepatotoxins. It would appear that it is the metabolism of carbohydrate which provides the organ with its increased resistance.[4]

It is of considerable interest to note that the administration of carbohydrate to patients with benign gastrointestinal disorders increased their hepatic glycogen stores considerably more than it did those of patients with gastrointestinal cancer or with bile duct obstruction. Conceivably, this difference could be due to (*a*) a decreased ability of patients with gastrointestinal cancer and bile duct obstruction (patients with considerable hepatic dysfunction) to absorb the administered glucose from their alimentary tracts, or (*b*) a decreased capacity of their livers to form or to store glycogen. Data obtained from routine oral glucose tolerance curves do not indicate that impaired absorption of carbohydrate from the alimentary tracts of patients with gastrointestinal cancer or obstructive biliary lesions would explain the abnormality.[13] On the other hand, it is known that these patients have a considerable degree of hepatic insufficiency [14] and that since it is a function of the liver to synthesize and to store glycogen, probably that function also has been damaged. The possibility should be entertained, however, that the limited capacity of the livers of these individuals to lay

down glycogen might be due to a derangement of those endocrine factors concerned with glycogenesis.[15]

It now appears that lipocaic exerts its lipotropic effect not only in experimental animals but also in human beings. Therefore, this material should prove useful in the preoperative preparation of patients with gastrointestinal cancer in order to decrease the susceptibility of their livers to the damage ordinarily sustained from surgical manipulation, operative anesthesia, and the necessary administration of those drugs detoxified by the liver.

The effect of the lipocaic apparently is not entirely due to its choline but may be due to its inositol content. Experiments now are under way to ascertain the lipotropic effects of (1) the amount of inositol equal to that in the 8 gm. dose of lipocaic, both when administered with and without choline, (2) smaller amounts of lipocaic, and (3) various proteins and amino acids. The results of these studies will be reported later.

Summary

1. The administration of glucose increased the hepatic glycogen stores significantly only in the patients with benign gastrointestinal disorders.

2. The preoperative oral administration of glucose to patients with gastrointestinal cancer, common bile duct obstruction, and with benign gastrointestinal lesions, significantly decreased the concentration of fat in their livers.

3. The administration of glucose did not affect significantly the concentration of hepatic protein in the three groups of patients studied.

4. The administration of lipocaic significantly decreased the content of fat in the livers of patients with gastrointestinal cancer. This effect could not be explained entirely by the choline content of the lipocaic, but may be due to its inositol content.

5. The ingestion of lipocaic did not affect significantly the concentrations of glycogen or protein in the livers of the three groups of patients studied.

6. It is suggested that patients who frequently come to operation with fatty infiltration of their livers (as do those with gastrointestinal cancer and bile duct obstruction) should be given preoperatively glucose and lipocaic in order to restore toward normal their altered hepatic chemical constitution. By these means it might prove possible to increase the resistance of their livers to damage by hepatotoxins.

Bibliography

1. Ariel, I. M., Abels, J. C., Murphy, H. T., Pack, G. T., and Rhoads, C. P.: Metabolic studies in patients with cancer of the gastrointestinal tract. VIII. The chemical composition of the liver, especially in patients with gastrointestinal cancer, Ann. Int. Med., 1940, xx, 570–579.

2. Greene, C. H.: Liver and biliary tract, a review for 1941, Arch. Int. Med., 1942, lxix, 691–714.

3. SOSKIN, S., and HYMAN, M.: Physiologic basis of intravenous dextrose therapy for diseases of the liver, Arch. Int. Med., 1939, lxiv, 1265–1270.
4. RAVDIN, I. S., VARS, H. M., THOROGOOD, E., SCHULTZ, J., and JOHNSON, J.: The liver glycogen and lipid concentrations following intravenous glucose administration and diet in the dog and man in the presence of liver damage, Ann. Surg., 1941, cxiv, 1018–1025.
5. MILLER, L. I., and WHIPPLE, G. H.: Chloroform liver injury increases as protein stores decrease: studies in nitrogen metabolism in these dogs, Am. Jr. Med. Sci., 1940, cxcix, 204–216.
6. BOYCE, F. F., and MCFETRIDGE, E. M.: Studies of hepatic function by the Quick hippuric acid test. III. Various surgical states, Arch. Surg., 1938, xxxvii, 443–445.
7. GREENE, C. H.: Liver and biliary tract: a review of the literature for 1942, Arch. Int. Med., 1943, lxxii, 563–578.
8. HAWKINS, W. B.: Liver and bile, *in* LUCK, J. M., and HALL, V. E.: Ann. Rev. Physiol., 1941, iii, 259–282, Ann. Reviews, Inc., Stanford University, California.
9. RALLI, E. P., RUBIN, S. H., and RINZLER, S.: The liver lipids in normal human livers and in cases of cirrhosis and fatty infiltration of the liver, Jr. Clin. Invest., 1941, xx, 93–97.
10. DRAGSTEDT, L. R., VAN PROHASKA, J., and HARMS, H. P.: Observations on a substance in pancreas (a fat metabolizing hormone) which permits survival and prevents changes in depancreatized dogs, Am. Jr. Physiol., 1936, cxvii, 175–181.
11. BEST, C. H., FERGUSON, G. C., and HERSHEY, J. M.: Choline and liver fat in diabetic dogs, Jr. Physiol., 1933, lxxix, 94–102.
12. GAVIN, G., and MCHENRY, E. W.: Inositol: a lipotropic factor, Jr. Biol. Chem., 1941, cxxxix, 485.
13. Unpublished data. These Laboratories.
14. ABELS, J. C., REKERS, P. E., BINKLEY, G. E., PACK, G. T., and RHOADS, C. P.: Metabolic studies in patients with cancer of the gastrointestinal tract. II. Hepatic dysfunction, ANN. INT. MED., 1942, xvi, 221–240.
15. LONG, C. N. H., KATZIN, B., and FRY, E. G.: The adrenal cortex and carbohydrate metabolism, Endocrinology, 1940, xxvi, 309–344.

Proc. Soc. Exp. Biol. Med. 1945 54: 157

14345

Metabolic Studies in Patients with Cancer of Gastro-Intestinal Tract. XV. Lipotropic Properties of Inositol.*

JULES C. ABELS, CLAUDIA W. KUPEL, GEORGE T. PACK, AND C. P. RHOADS.

From the Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York City.

Recent studies from this hospital have demonstrated that the livers of patients with gastro-intestinal cancer almost uniformly are infiltrated with fat.[1] In contrast to this finding, normal fat contents were found in the livers of 11 patients with carcinoma of the gastro-intestinal tract who each received 8 g of lipocaic† during the night prior to laparotomy. The average hepatic fat concentration of this latter group was only 0.46 that of the control untreated patients.[2]

Lipocaic, however, is a crude mixture which contains significantly large amounts of choline and inositol,† both of which are lipotropic alone.[3,4] In experimental animals, the lipotropic effects of lipocaic apparently are not due to its choline alone,[5] and this observation has been confirmed here for human subjects.[2] On the other hand, when about 4 times the amount of inositol present in the 8 g of lipocaic was administered to a group of 8 patients with gastro-intestinal cancer, with but one exception the concentrations of fat in their livers were found to be within normal limits. The average lipid value was 0.42 that of the control group.

TABLE I.
Concentration of Fat in Livers of Patients with Gastro-intestinal Cancer Who Received Preoperatively 280 mg of Inositol.

Patient	Disorder	Conc. of hepatic fat, g per 100 g wet tissue
J.K.	Carcinoma oesophagus	7.2
R.H.	" stomach	8.7
O.S.	" colon	7.1
M.S.	" stomach	10.3
M.T.	" colon	7.8
W.R.	" stomach	5.7
H.J.	" "	5.0
F.M.	" "	15.9
G.M.	" "	5.9
M.J.	" "	8.9
	Avg	8.25

Because it seemed probable then that the inositol alone might account for the physiologic effects of the lipocaic, another group of 10 patients with carcinoma of the gastro-intestinal tract was given during the last 10 preoperative hours only that amount of inositol (280 mg) contained in the effective dose of lipocaic. The clinical and chemical methods used have been described in a preceding communication of this series.[1] The results obtained from the ingestion of the smaller amounts of inositol indicate that in the patients studied, the lipotropic properties of the lipocaic could be accounted for by its content of inositol alone (Tables I and II). Of the values of hepatic lipid, only one was significantly elevated, and the average of the group was only 0.50 that of the control group.

* The authors gratefully acknowledge the support of the National Cancer Institute.

† The lipocaic used in this investigation was supplied through the courtesy of Eli Lilly & Co., Indianapolis, Ind., and the inositol through the kindness of Lederle & Co., Pearl River, N.Y. The concentration of choline and of inositol in the material used was found by analyses to be 2.6 and 3.5 g % respectively. These analyses were made by Dr. W. Wooley of the Rockefeller Institute for Medical Research, New York City, and by the Laboratories of the Standard Brands, Inc., New York City.

[1] Ariel, I. M., Abels, J. C., Murphy, H. T., Pack, G. T., and Rhoads, C. P., *Ann. Int. Med.*, in press.

[2] Abels, J. C., Ariel, I. M., Murphy, H. T., Pack, G. T., and Rhoads, C. P., *Ann. Int. Med.*, in press.

[3] Best, C. H., Ferguson, G. A., and Hershey, J. M., *J. Physiol.*, 1933, **79**, 94.

[4] Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, 1941, **139**, 485.

[5] Dragstedt, L. R., *J. Am. Med. Assn.*, 1940, **114**, 29.

TABLE II.
Average Concentration of Fat in the Livers of Patients with Gastro-intestinal Carcinoma Who Were Fasted and of Those Given Various Dietary Supplements Preoperatively.

No. of patients	Preoperative supplement	Avg conc of hepatic fat, g per 100 g wet tissue	% reduction of hepatic lipid effected by supplement
28	None	16.4	—
11	8 g lipocaic	8.0	51
7	3 g choline chloride	11.2	39
8	1200 mg inositol	6.9	58
10	280 " "	8.2	50

J. Biol. Chem. 1916; 25:391-397

THE UTILIZATION OF INOSITE IN THE DOG.*

By R. J. ANDERSON.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, and the Institute of Physiology, University College, London.)

(Received for publication, May 6, 1916.)

INTRODUCTION.

In connection with the investigations concerning the occurrence and chemical properties of phytin and inosite phosphoric acids[1] and the physiological rôle of these substances, we have undertaken a few experiments to determine the fate of inosite and its utilization in the animal organism. The previous work on phytin in this laboratory[2] concerned itself chiefly with the physiological relation of the phytin phosphorus to the metabolism. In the present paper we deal with the organic radical, inosite, of the phytin molecule.

The wide distribution of inosite in the vegetable and animal kingdoms has led to many investigations concerning the physiological function of this substance in the animal organism. Since inosite was first discovered in muscle by Scherer[3] it has been found in practically all the tissues and fluids of the body[4] and it

* Read before the Section of Biological Chemistry at the Urbana meeting of the American Chemical Society, April 20, 1916. The experimental work herein reported was carried out in the Institute of Physiology, University College, London.

[1] Anderson, R. J., *J. Biol. Chem.*, 1912, xi, 471; xii, 97, 447; 1912–13, xiii, 311; 1914, xvii, 141, 151, 165, 171; xviii, 425, 441; 1915, xx, 463, 475, 483, 493; *New York Agric. Exp. Station, Technical Bull.*, 19, 21, 22, 25, 1912; 32, 36, 1914; 40, 1915.

[2] Jordan, W. H., Hart, E. B., and Patten, A. J., *Am. J. Physiol.*, 1906, xvi, 268; *New York Agric. Exp. Station, Technical Bull.*, 1, 1906. Rose, A. R., *New York Agric. Exp. Station, Technical Bull.*, 20, 1912.

[3] Scherer, *Ann. Chem.*, 1850, lxxiii, 322.

[4] A review of the literature will be found in Jacobson, P., and Meyer, V., *Lehrbuch organ. Chem.*, 1902, ii, pt. i, 807.

appears to be a normal constituent of the urine.[5] In the vegetable kingdom, inosite is also widely distributed, occurring free in many plants and, particularly, conjugated with phosphoric acid as phytin or inosite hexaphosphoric acid[6] in seeds, roots, or tubers.

The earlier investigators[7] sought to establish some relation of inosite to the carbohydrates and to the elimination of sugar in diabetes. Külz[8] in an extensive series of experiments examined both normal and diabetic subjects. He found normal human urine practically free from inosite, but from the urine of six normal males after excessive drinking of beer or wine he obtained from 0.4 to 0.9 gm. of inosite. After feeding from 30 to 50 gm. of inosite to normal individuals he was able to recover only from 0.2 to 0.5 gm. of inosite from the urine. In the case of diabetics the same quantity of inosite gave practically the same result and there was no increase in the elimination of sugar. He thought for this reason that inosite might be utilized in the diabetic organism as a source of energy in place of sugar. By experiments on rabbits he also showed that inosite did not give rise to the formation of glycogen.

These studies were later continued by Mayer.[9] His results confirm those reported by Külz concerning the non-formation of glycogen from inosite. After feeding from 2 to 15 gm. of inosite *per os* to rabbits, only small amounts, from 2 to 2.4 per cent of the substance, were recovered from the urine. When it was given subcutaneously, however, he found that from 26 to 51 per cent of the inosite was eliminated unchanged in the urine. In addition to the inosite small quantities of lactic acid[10] were sometimes isolated from the urine after the inosite had been given subcutaneously.

[5] Hoppe-Seyler's Handb. Phys.- u. Path.-Chem. Analyse, Berlin, 7th edition, 1903, 220. Starkenstein, E., *Z. exp. Path. u. Therap.*, 1908-09, v, 378.

[6] A review of literature is given by Rose, A. R., *Biochem. Bull.*, 1912–13, ii, 21.

[7] See Starkenstein,[5] for a review of the literature.

[8] Külz, E., Sitzungsberichte Ges. z. Beförd. ges. Naturwissen., Marburg, 1876; Beitr. Path. u. Therap. Diabetes Mellitus, 1874, i.

[9] Mayer, P., *Biochem. Z.*, 1907, ii, 393.

[10] Mayer, *Biochem. Z.*, 1908, ix, 533.

Results very similar to the above were reported about the same time by Starkenstein.[5] About 5 per cent of the inosite given *per os* to rabbits was recovered in the urine. When the substance was injected subcutaneously about 42 per cent and after intravenous injections about 50 per cent of the inosite was eliminated in the urine. This author concludes that inosite is a normal cell constituent; that it is only slightly oxidized in the body because a large percentage of it is eliminated unchanged in the urine after being given in subcutaneous or intravenous injections, and that disappearance of inosite given *per os* is not permissible as proof of its being oxidized by the body since it may be destroyed by bacteria in the intestine.

It is evident from the work which has been done on this subject that inosite given *per os* is either largely destroyed by bacteria in the intestine or else it is stored or oxidized in the body, because very small amounts of it are eliminated in the urine. In the hope of throwing further light upon the fate of inosite in the animal organism we have carried out some respiration experiments with a dog which, in a fasting condition, was fed inosite *per os* just previous to being placed in the respiration apparatus. If inosite were oxidized in the body in the same manner as the carbohydrates or dextrose, for instance, then a rise in the respiratory quotient should be observed. The experiments have shown, however, that there was no appreciable rise in the respiratory quotient after giving 10 gm. of inosite. It is evident, therefore, that inosite is not oxidized in the dog in the manner of dextrose, and if oxidation or other changes take place they do so slowly and in such a way as not to affect the respiratory quotient during the first 2 hours following its ingestion.

It was observed, about 2 or 3 hours after the inosite had been given, that considerable diarrhea set in. Unfortunately it was not possible to separate the urine and the liquid excreta; these were therefore examined together. In one case, however, the liquid feces were voided outside of the metabolism cage and the urine was collected quite clear. When this clear urine was examined it was found to contain but 0.4 gm. of inosite. This shows, as in the case of man reported by Külz[8] and rabbits reported by Mayer[9] and Starkenstein,[5] that but small amounts of inosite are eliminated through the kidneys of the dog. When

the urine was mixed with liquid feces, however, very large quantities of inosite were obtained, and in one case as much as 77 per cent of the amount given was recovered from the mixed excreta.

EXPERIMENTAL.

The inosite used in this work was prepared from crude phytin by hydrolyzing this substance with dilute sulfuric acid in an autoclave. It was carefully purified by repeated recrystallization from water. The snow-white product finally obtained was free from ash and melted at 220° (uncorrected).

A small bitch weighing 5,700 gm. was selected for these experiments and trained for use in the respiration apparatus. The animal was kept in a metabolism cage and fed once a day, in the evening, on cooked meat and dog biscuit, but during the period when inosite was given meat was the sole food.

The Benedict respiration apparatus described by Moorhouse, Patterson, and Stephenson[11] was used. The carbon dioxide and oxygen measurements were made as there described, and for particulars we refer to the above paper.

The animal was fed about 5 p.m., and the respiration observations were made about 18 hours afterwards. The quotients in this fasting condition are given in Table I. The figures obtained lie within the limits usually observed under these conditions.

TABLE I.

Fasting Respiratory Quotients.

Date.	Length of period.	CO_2 per minute.	O_2 per minute.	R. Q.	Remarks.
	min.	*cc.*	*cc.*		
Feb. 11	42	40.35	54.61	0.739	Usual movements.
" 12	64	44.62	58.65	0.760	Somewhat restless.
" 15	58	42.03	55.72	0.754	" "
" 24	46	35.95	45.65	0.787	Very quiet.

The inosite was given dissolved in about 70 cc. of warm water. This solution was taken at first just as readily as one of glucose,

[11] Moorhouse, V. H. K., Patterson, S. W., and Stephenson, M., *Biochem. J.*, 1915, ix, 176.

but after a while the inosite solution was absolutely refused and it had to be given by the stomach tube. A few minutes after the inosite had been given the animal was placed in the respiration apparatus, and the observation periods varied from 1½ to about 2 hours. It will be noticed by referring to the figures in Table II that the respiratory quotients during these periods are slightly

TABLE II.

Respiratory Quotients after Giving 10 Gm. of Inosite.

Date.	Length of period.	CO_2 per minute.	O_2 per minute.	R. Q.	Remarks.
	min.	*cc.*	*cc.*		
Feb. 11	90	45.18	57.73	0.782	Restless.
" 12	126	44.72	56.50	0.791	"
" 15	123	41.13	56.50	0.780	"
" 17	111	43.38	58.08	0.747	"

higher than those in the fasting condition (Table I). The difference is very slight, however, and it is doubtful if the small rise in the respiratory quotient is due to oxidation of the inosite. The animal showed signs of discomfort during the inosite periods and was more or less restless, consequently the oxygen measurement was more difficult. Diarrhea usually set in a short time after the animal was removed from the respiration apparatus.

For comparison with the quotients tabulated above some figures after feeding glucose are given in Table III.

TABLE III.

Respiratory Quotients after Feeding Glucose.

Date.	Glucose.	Length of period.	CO_2 per minute.	O_2 per minute.	R. Q.	Remarks.
	gm.	*min.*	*cc.*	*cc.*		
Feb. 9	20	63	59.77	60.84	0.982	Restless.
" 18	10	78	65.20	66.02	0.989	"

In Table IV are given the amounts of inosite[12] recovered from the urine and liquid feces. In this connection it must be stated that only the liquid portion of the excreta which collected in the bottle underneath the metabolism cage was analyzed for inosite.

[12] The inosite was isolated by the method of P. Mayer.[9]

TABLE IV.

Inosite Recovered from Urine and Liquid Feces.

Experiment.	Inosite given.	Inosite recovered.		Remarks
	gm.	*gm.*	*per cent*	
1	10	0.40	4.0	Urine clear.
2	10	4.00	40.0	" plus feces.
3	10	1.27	12.7	" " "
4	10	2.25	22.5	" " "
5	10	6.30	63.0	" " "
6	10	7.70	77.0	" " "
1st day	None.	0.20		" clear.
2nd "				
3rd "	"	Trace.		" "

The variation in the amounts of inosite recovered in the first four experiments is due no doubt to the fact that in these periods the dog was used for the respiration observations and shortly after being removed from the respiration apparatus diarrhea set in and these liquid stools were lost. During the fifth and sixth experiments, however, the dog was kept in the metabolism cage the whole time and all the liquid feces and the urine were collected together.

After the sixth experiment the clear urine was collected for the next 3 days, and analyzed for inosite. By referring to the table it will be noticed that the combined urine of the 1st and 2nd days contained only 0.2 gm. of inosite while on the 3rd day a mere trace was obtained. This shows that practically all the inosite given is eliminated within 24 hours; evidently, therefore, either none or very little of it can be stored in the body.

Since the clear urine (Table IV) contained little and the urine plus liquid feces contained much inosite, it would seem that by far the greater portion of the substance was not absorbed but that it was eliminated through the bowel. In order to determine to what extent inosite is absorbed in the intestine the following experiment was made. A section about 2 feet long of the small intestine of a dog was exposed[13] and washed out with physiological salt solution. One end was ligatured, a solution of 3 gm. of inosite in 50 cc. of warm water was introduced, and the other

[13] The operation was performed by Professor E. H. Starling.

end was also ligatured. The whole was replaced in the abdominal cavity, the incision loosely closed, and the animal kept under chloroform for 2 hours. On removing the section of the bowel it was quite distended and contained 90 cc. of liquid. From this liquid 2.95 gm. of inosite were isolated; *i.e.*, the substance was recovered practically quantitatively.

CONCLUSIONS.

Inosite is not utilized to any extent by the dog. It is not stored or oxidized in the body but the greater portion of it is excreted unchanged. When given at the rate of 2 gm. per kg. of body weight it does not cause a rise in the respiratory quotient.

As much as 77 per cent of the amount given may be recovered from the excreta.

Inosite is absorbed very slowly from the intestine, hence it causes a more or less severe diarrhea. As a result it is largely eliminated with the feces and only a small portion is excreted by the kidneys.

The author acknowledges his indebtedness to Professer E. H. Starling for help and advice in carrying out the above e periments, and to Dr. V. H. K. Moorhouse for assistance in using the respiration apparatus.

J. Biol. Chem. 25:399-407;1916

THE UTILIZATION OF INOSITE IN THE ANIMAL ORGANISM. THE EFFECT OF INOSITE UPON THE METABOLISM OF MAN.*

By R. J. ANDERSON and A. W. BOSWORTH.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva.)

(Received for publication, May 6, 1916.)

INTRODUCTION.

The objects of this investigation were to determine whether the ingestion of inosite has any noticeable influence upon the metabolism of man measurable in terms of the nitrogen and phosphorus excretion, and also the fate of inosite in the human body and its channels of elimination.

It has been shown by one of us[1] that inosite is not utilized to any extent by the dog, that it acts as a strong purgative, and that the greater portion is eliminated unchanged through the bowel.

That inosite causes more or less severe diarrhea in both man and animals when given in large quantities has been observed previously by Külz[2] who thought that the diarrhea was caused by the transformation of the inosite into lactic acid in the intestine. In normal or diabetic human subjects from 30 to 50 gm. of inosite per day caused considerable diarrhea and only from 0.2 to 0.5 gm. of the inosite was recovered from the urine. This author does not report the examination of the feces of his subjects for inosite. In the case of rabbits the above author found that inosite caused diarrhea and only small quantities of the substance were recovered from the urine. Results very similar to the above

* Read before the Section of Biological Chemistry at the Urbana meeting of the American Chemical Society, April 29, 1916.

[1] Anderson, R. J., *J. Biol. Chem.*, 1916, xxv, 391.

[2] Külz, E., Sitzungsberichte Ges. z. Beförd. ges. Naturwissen., Marburg, 1876.

have been reported by Mayer[3] and Starkenstein[4] who both used rabbits as subjects.

Of the previous investigations concerning the physiological rôle of inosite in man those of Külz[2] are probably the most thorough; he had an unusually large quantity of inosite at his disposal for experimental purposes, having prepared about a pound of it from green beans. The high price and the small available quantities of inosite have presented difficulties in carrying out investigations with this substance. For the present work we prepared several hundred grams of pure inosite as described on page 394.

Our results confirm those of previous investigators that inosite given *per os* disappears and only small quantities are eliminated in the urine. Külz[2] recovered only about 0.9 per cent of the inosite given from the urine of human subjects. Our figures, however, are about ten times greater; *i.e.*, we recovered from the urine nearly 9 per cent of the ingested inosite. Careful examination of the feces was made but we failed to obtain a trace of inosite. Consequently the urine is the only channel of elimination of inosite in man.

In what manner the balance, about 91 per cent, of the inosite is utilized or destroyed is uncertain. It has been shown by the investigations of Meillère[5] and also by Starkenstein,[4] that inosite is destroyed by the colon bacillus. It has also been shown by Hilger[6] and Vohl[7] that inosite is transformed into lactic acid by the bacteria found in putrid cheese. Experiments by Starkenstein[4] indicate that inosite is destroyed by autolytic ferments of muscles and the liver with the simultaneous increase of lactic acid.

Our results do not show whether the inosite was destroyed by bacteria in the intestine or underwent oxidation in the body. If the inosite was changed into lactic acid it is evident that only traces of it were excreted as such, because there was no appreciable increase of ammonia in the urine (Table III). At present we can only record the fact that of the inosite given only about 9 per cent escaped destruction and was recovered from the urine.

[3] Mayer, P., *Biochem. Z.*, 1907, ii, 393.
[4] Starkenstein, E., *Z. exp. Path. u. Therap.*, 1908-09, v, 378.
[5] Meillère, G., *Compt. rend. Soc. biol.*, 1907, lxii, 1096.
[6] Hilger, *Ann. Chem.*, 1871, clx, 333.
[7] Vohl, H., *Ber. chem. Ges.*, 1876, ix, 984.

It is evident from the data presented in the experimental part that, except for the increased excretion of creatinine in the after-period, the ingestion of inosite at the rate of about 0.5 gm. per kg. of body weight has no appreciable influence upon the metabolism of man.

EXPERIMENTAL.

To determine the channels of elimination of inosite in man we carried out the following preliminary experiment. One of us (B.) received 30 gm. of inosite dissolved in water in three equal portions during the day. The following morning a solution of 10 gm. of inosite was given. About 4 hours after the first 10 gm. of inosite were taken a very loose watery stool was passed and during the 24 hours there were three more very watery stools. The second day the stools were almost of normal consistency. During the first and second days the subject complained of an uneasy or uncomfortable feeling and of a peculiar sensation in the chest and abdominal muscles. This feeling disappeared towards the end of the 3rd day and on the 4th day he felt normal again.

The urine and feces were collected in 24 hour periods and examined for inosite. For the isolation of the inosite we used the method of Mayer[3] which is briefly as follows: The urine was evaporated to about one-fourth of its volume and precipitated with excess of lead acetate. After standing a short while the precipitate was filtered off on a Buchner funnel and washed with water. The filtrate was heated to boiling and precipitated with excess of basic lead acetate and finally rendered strongly alkaline with concentrated ammonia. After standing for 24 hours the precipitate was filtered on a Buchner funnel and washed with water. It was then suspended in water and decomposed with hydrogen sulfide. After removing the lead sulfide, the filtrate was evaporated on the water bath almost to dryness. The residue was taken up in a little hot water, decolorized with animal charcoal, again evaporated to small bulk, transferred to an Erlenmeyer flask, and brought to crystallization by the addition of about ten volumes of absolute alcohol. After the greater portion of the inosite had crystallized out ether was added until the solution turned cloudy and the whole was allowed to stand in

the ice box over night. The crystals were then filtered off, washed with alcohol and ether, dried in the air, and weighed.

The feces were examined as follows: The fresh material was stirred up with water until of uniform consistency. Lead acetate was then added and after standing for some time it was filtered and washed. The filtrate was evaporated, precipitated with basic lead acetate, etc., as mentioned above.

The amount of inosite recovered in the excreta is given in Table I.

TABLE I.

Day.	Inosite given.	Inosite recovered from the urine.		Inosite recovered from the feces.
	gm.	*gm.*	*per cent*	
1	30	2.6	8.66	None.
2	10	1.1	11.00	"
3	None.	Trace.		"

The results show that in man a small quantity of the ingested inosite is eliminated in the urine and that the feces contain none; also that it is either absorbed and oxidized in the body or else destroyed in the intestine, and that the excretion of unchanged inosite is complete in 24 hours, because on the 3rd day the urine contained only a trace of the substance. We find that the quantity of inosite excreted by the kidneys is about ten times greater than found by Külz. This is probably due to the improved method of isolating the substance.

In a second more extensive experiment we have sought to discover if the ingestion of inosite produced any change or disturbance in the metabolism. For this purpose we selected a period of 8 days during which the food of the subject (B.) was carefully regulated. Unfortunately we failed to maintain any control of the food intake during the days following the actual experiment or after-period. The results are vitiated also by the fact that the period over which our experiment extended was not of sufficient length to bring the subject to a condition of nitrogen equilibrium and that we analyzed only one sample of each article of food. Since it was a liberal and mixed diet consisting of meat, eggs, milk, potatoes, bread, etc., it is probable that the actual intake during the whole period was different from what we figured from the result of one analysis. Despite these drawbacks we feel justified in publishing briefly the results obtained because these results are negative; *i.e.*, our observations have failed to reveal any noteworthy or striking change in the general metabolism as a result of the ingestion of inosite.

During this second experiment which was begun a few months after the one reported above, we began the inosite period by giving only 15 gm. of the substance the 1st day. On the 2nd and subsequent days 30 gm. of inosite were given. In this way the uncomfortable diarrhea was avoided. The stools were more frequent and softer than usual, but towards the end of the period they were nearly of normal consistency. There were no feelings of discomfort, in fact the subject felt practically normal throughout the experiment, performing his laboratory work in the usual manner.

The total nitrogen and phosphorus balance, with the caution mentioned above, is given in Table II. The difficulty of bringing man to a nitrogen equilibrium and the fact that the diet gave a nitrogen intake quite a little below the usual average daily intake of the subject makes the minus nitrogen and phosphorus balance have no special significance.

TABLE II.

Total Nitrogen and Phosphorus Balance.

Day.	Preliminary period.				Inosite period.			
	1	2	3	4	5	6	7	8
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Nitrogen intake....	15.254	15.483	11.781	12.131	12.880	11.355	11.151	11.249
Nitrogen excretion.	15.018	13.602	14.233	12.667	13.943	11.682	13.147	11.821
Phosphorus intake....	1.832	1.708	1.362	1.384	1.592	1.318	1.323	1.330
Phosphorus excretion.	2.1267	1.6626	1.8688	1.5634	1.8469	1.4029	1.5119	1.4680

		gm.		*gm.*
Total nitrogen intake,	preliminary period,	54.649;	inosite period,	46.635
" " excretion,	" "	55.520;	" "	50.593
Minus nitrogen balance,		0.871;		3.958
" phosphorus intake,	preliminary period,	6.286;	inosite period,	5.563
" " excretion,	" "	7.2215;	" "	6.2297
Minus phosphorus balance,		0.9355;	" "	0.6667

Figures covering the elimination of nitrogen in the urine are tabulated in Table III. There was a considerable rise in the creatinine output which began on the last day of the inosite period and extended through the whole of the after-period, the first 2 days of the after-period showing a marked increase in creatinine elimination. During the after-period the subject ate the same diet as during the rest of the experiment but the food was not accurately weighed. In view of this fact, the increased creatinine output during the after-period would seem to be an important observation and a matter worthy of further study.

The uric acid, creatinine, and ammonia were determined by the Folin methods.

TABLE III.

Analysis of the Urine. Nitrogen Elimination.

Preliminary Period.

Day	1	2	3	4
Volume of urine, cc.	1,420	1,480	1,170	710
Specific gravity	1.019	1.015	1.020	1.028
Total nitrogen, gm.	12.908	12.802	12.273	11.204
Uric acid, gm.	0.636	0.658	0.585	0.546
Creatinine, gm.	1.198	1.199	1.050	1.150
Ammonia nitrogen, gm.	0.965	0.864	0.959	0.899

Inosite Period.

Day	5	6	7	8
Volume of urine, cc.	770	810	1,450	900
Specific gravity	1.026	1.026	1.016	1.022
Total nitrogen, gm.	11.504	9.914	11.296	9.234
Uric acid, gm.	0.550	0.463	0.520	0.692
Creatinine, gm.	1.299	1.010	1.068	1.448
Ammonia nitrogen, gm.	1.089	0.868	1.125	0.918

After-Period.

Day	9	10	11	12
Volume of urine, cc.	1,175	1,390	1,550	1,070
Specific gravity	1.022	1.022	1.016	1.023
Total nitrogen, gm.	12.009	13.232	8.680	8.988
Uric acid, gm.	0.628	0.731	0.620	0.767
Creatinine, gm.	1.729	2.183	1.141	1.332
Ammonia nitrogen, gm.	0.940	0.970	0.639	0.6[illegible]

The phosphorus elimination in the urine is recorded in Table IV. The total phosphorus was determined after destroying the organic matter by the Neumann method. The inorganic phosphorus is that quantity which was directly precipitated by ammonium molybdate after acidifying the urine with nitric acid and adding ammonium nitrate. The organic phosphorus was found by difference. It will be noticed by referring to the table that the organic phosphorus varied from 0 to 0.0178 gm. with an average of 0.0102 gm. per day. This agrees with the average quantity of organic phosphorus usually found in normal urine.[5]

TABLE IV.

Elimination of Phosphorus in the Urine.

Preliminary Period.

Day.	1		2		3		4	
Volume of urine, cc.	1,420		1,480		1,170		710	
	per cent	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*
Total phosphorus	0.0835	1.1857	0.0843	1.2476	0.0946	1.1068	0.1433	1.0174
Inorganic phosphorus	0.0826	1.1729	0.0831	1.2298	0.0946	1.1068	0.1413	1.0032
Organic phosphorus	0.0009	0.0128	0.0012	0.0178	0	0	0.0020	0.0142

Inosite Period.

Day.	5		6		7		8	
Volume of urine, cc.	770		810		1,450		900	
	per cent	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*
Total phosphorus	0.1370	1.0549	0.1174	0.9509	0.0742	1.0759	0.1030	0.9270
Inorganic phosphorus	0.1358	1.0456	0.1165	0.9436	0.0738	1.0701	0.1014	0.9126
Organic phosphorus	0.0012	0.0093	0.0009	0.0073	0.0004	0.0058	0.0016	0.0144

The amount of nitrogen and phosphorus excreted in the feces is given in Table V. A notable rise in the nitrogen excretion is observed during the inosite period. This was no doubt due to the more frequent stools and consequent less complete absorption in the intestine. Since it was shown in the preliminary experi-

[5] For a review of the literature see Forbes, E. B., and Keith, M. H., *Ohio Agric. Exp. Station, Technical Bull.*, 5, 1914, 190.

TABLE V.

Nitrogen and Phosphorus Excretion in the Feces.

Day.	Preliminary period.				Inosite period.			
	1	2	3	4	5	6	7	8
Nitrogen, gm.	2.110	0.800	1.960	1.463	2.439	1.768	1.851	2.587
Phosphorus, gm.	0.941	0.415	0.762	0.546	0.792	0.452	0.436	0.511

ment that inosite was eliminated only in the urine the feces were not examined this time for this substance.

In Table VI the daily intake of inosite and the quantities recovered from the urine are recorded. The same method of isolation was used as before. The inosite was excreted in about the same proportion as in the preliminary experiment. The total intake amounted to 105 gm. of inosite and only about 9 gm. were recovered. The balance, 96 gm., was either oxidized in the body or destroyed by bacteria in the intestine. Evidently the inosite could not have been stored in the body because the elimination ceases with the intake.

TABLE VI.

Inosite Intake and Excretion.

Day.	5	6	7	8	9	10	11	12
Volume of urine, cc.	770	810	1,450	900	1,175	1,390	1,550	1,070
Inosite intake, gm.	15	30	30	30	None.	None.	None.	None.
Inosite excretion, gm.	0.38	2.5	3.19	2.5	0.47	Trace.	Trace.	Trace.

Total intake, 105 gm. of inosite.
Inosite recovered, 9.04 gm. or 8.6 per cent.

SUMMARY.

When inosite is taken at the rate of about 0.5 gm. per kg. of body weight per day it produces some diarrhea at first or frequent soft stools. After a few days the stools, although more frequent than usual, are nearly of normal consistency.

Except for the increased excretion of creatinine in the after-period, for which we can now offer no explanation, we find that the ingestion of inosite has no marked or appreciable effect upon the general metabolism of man.

About 9 per cent of the inosite taken *per os* is eliminated unchanged in the urine, but none in the feces. In what manner the balance, or about 91 per cent, of the inosite is utilized we have not been able to determine.

Ann. Internal Med. 20:570-578; 1944

METABOLIC STUDIES IN PATIENTS WITH CANCER OF THE GASTROINTESTINAL TRACT. VIII. THE CHEMICAL COMPOSITION OF THE LIVER, ESPECIALLY IN PATIENTS WITH GASTROINTESTINAL CANCER*

By IRVING M. ARIEL, M.D.,† JULES C. ABELS, M.D.,‡ HELEN T. MURPHY, PH.D., GEORGE T. PACK, M.D., and C. P. RHOADS, M.D., F.A.C.P.,
New York, N. Y.

INTRODUCTION

PREVIOUS studies from this hospital have demonstrated that patients with gastrointestinal cancer frequently have several abnormalities of hepatic function.[1, 2] The existence of these disorders appears to be due to the presence of the neoplasm, for when it is removed the functional capacity of the liver improves.[2] The occurrence of hepatic insufficiency in these patients seldom has been found to be associated with those morphological alterations of the liver[3] commonly observed in patients who suffer from chronic alcoholism or cirrhosis.[4]

The possibility existed that even though the livers of patients with gastrointestinal tract cancer were morphologically intact, their chemical compositions might be abnormal. If this were the case it might be of clinical significance, since it would provide a possible explanation for the hepatic insufficiency shown by these patients, and would suggest an effective method for the treatment of this insufficiency. The last possibility is based upon the fact that the administration of certain dietary constituents to experimental animals with livers which contain abnormal amounts of fat and glycogen has been followed by a return of those organs to a normal chemical state and increased functional capacity.[5, 6]

The chemical composition of the livers of patients with cancer of the gastrointestinal tract has been studied and the effects of the administration of dietary factors observed. The results of these studies form the subject of this and the subsequent communication.

MATERIAL

Liver biopsies were obtained from one test and two control groups of patients. The test group consisted of 18 patients with gastrointestinal cancer. Of these, one had carcinoma of the terminal esophagus, 15 of the

* Received for publication June 9, 1943.
From The Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York City.
The authors gratefully acknowledge a grant from the National Cancer Institute.
† Trainee in diagnosis and treatment, National Cancer Research Institute.
‡ Finney-Howell Fellow.

stomach, and two of the colon. Three patients had extension of the tumor to the liver.

The first control group comprised four patients: one with carcinoma of the head of the pancreas, one with toxic hepatitis, and two with chronic cholecystitis with impacted calculi in the common bile duct. This group was studied because their disorders are commonly associated with marked hepatic insufficiency and morphologic damage.[5, 6]

The second control group consisted of four patients who were operated upon for non-neoplastic lesions of the gastrointestinal tract. One had gastritis, two gastric ulcer, and one multiple duodenal ulcers. None suffered from pyloric stenosis. Patients with the disorders included in this group do not show any significant degree of hepatic dysfunction.

In all the diagnoses were confirmed by microscopic examination of the biopsied lesions.

Methods

A. Clinical: All of the 26 patients studied were hospitalized for from three to eight days before they were operated upon. They were given daily at least 2500 ml. fluid and a diet of from 2,000 to 3,000 calories. The ratio of carbohydrate: fat: protein in their diet was 5 : 1 : 1. Only clear fluids were given during the evening meal on the day before operation, and nothing thereafter. None received glucose or saline preoperatively.

Spinal anesthesia was used in all instances. The liver biopsies for chemical and microscopic study were obtained as soon as the peritoneal cavity was exposed. Specimens which weighed from 0.8 to 1.5 gm. were removed from the liver edge. Bleeding was controlled easily by the approximation of the cut surfaces by silk mattress sutures. As soon as the tissue was excised the fibrous capsule was removed and the remaining tissue was given to a laboratory assistant in the operating room. This was rapidly blotted and about 0.3 gm. placed in warm 30 per cent KOH for the glycogen determination. About 0.5 gm. was placed in a tared weighing bottle, weighed, and used for the determination of nitrogen and lipid. A small portion was fixed in formalin for histological study.

B. Chemical: 1. The glycogen was determined by the method of Good, Kraemer and Somogyi[7] as modified by Nutter.[8] The sugar content in aliquots of the hydrolysate was measured by the Shaffer and Somogyi technic.[9]

2. The protein and non-protein nitrogen fractions of the liver tissue were determined in fine aqueous suspensions. These were prepared by grinding about 300 mg. of the tissue with 10 ml. of water in an all glass homogenizer. The separation of the "protein" * and non-protein nitrogen fractions of this suspension was effected by the method of Robinson, Price, and Hogden.[10]

* It must be emphasized that the hepatic albumin and globulin fractions as presented in this study represent only those proteins which can be separated by precipitation with 22 per cent Na_2SO_4 at 37° C. This does not imply, therefore, that these protein fractions exist in the liver nor are they identical with serum albumin or globulin.

3. The liver total lipid was determined by the technic of Van Slyke et al.[11]

4. The protein concentration in the serum was ascertained by the method of Koch et al.[12]

Results

The results of this study will be presented in three sections: Those which deal with the hepatic content of glycogen, of fat, and of protein.

A. Glycogen: The glycogen content of normal human livers probably is not known, for most analyses have been made on postmortem material. The pre-agonal state probably alters the chemical composition of liver tissue,[13] and postmortem autolysis of hepatic glycogen occurs very soon after death. The postmortem glycogen levels in the liver tissue of normal individuals who died suddenly are reported to range from 2 to 8 gm. per cent.[14, 15] The values during life probably are somewhat higher. Although some measurements of glycogen have been made on liver tissue obtained at laparotomy,[16, 17] the patients were subject to disorders associated with hepatic dysfunction.

In the 18 patients studied with gastrointestinal cancer the glycogen content ranged from 1.0 to 8.3 gm., and averaged 2.91 gm. per cent. In the first control group, that of patients with hepatic dysfunction due to toxic hepatitis or biliary tract obstruction, the liver glycogen ranged from 1.3 to 3.1 gm. and averaged 2.62 gm. per cent. These values are probably abnormally low and are in good agreement with those reported by Goldschmidt et al.[18] for patients with liver damage. The four patients with benign lesions of the alimentary tract who form the second control group were found to have from 1.3 to 3.6 gm. of hepatic glycogen with an average of 2.28 gm. per cent. These patients were not found to have any hepatic dysfunction, nor were their livers morphologically abnormal. It appears either that the average value of 2.28 gm. per cent hepatic glycogen found in the patients with benign lesions is the amount present in the normal human liver, or that benign gastrointestinal disease is associated with abnormally low concentrations.

It is possible that the usual 10 hour preoperative fast decreased the glycogen stores in the livers of all the patients studied. Probably more revealing and reliable information could be obtained by the measurement of hepatic glycogen after the patients had received preoperatively large amounts of glucose. By these measurements an index of the ability of these patients to fabricate and store glycogen in the liver could be determined. Data of this nature now have been obtained and are included in the subsequent communication.[19]

B. Fat: The normal fat content of human livers is not definitely known. Values of from 2.4 to 8.5 gm. per cent lipid have been found[18] in the livers of normal individuals who were autopsied within several hours after death as a result of trauma. Although the hepatic content of fat probably does not change significantly within so short a time after exitus, it is admitted[18]

that the pre-agonal state may alter the content of fat as well as that of other constituents.

The average concentration of total lipid in the livers of the 18 patients with gastrointestinal cancer was 17.43 gm. per cent, and the range from 5.3 to 35.0 gm. per cent. Of the 18 values, 16 were higher than the highest "normal" value of 8.5 gm. per cent.[18] Thus, the livers of patients with gastrointestinal cancer frequently are infiltrated with fat, even though microscopic examination of the liver tissue fails to suggest that abnormality (when prepared by routine methods not specifically designed to demonstrate fat).

The lipid contents of the livers of patients with gastrointestinal cancer were as great as those of the patients in the first control group, those with carcinoma of the head of the pancreas, toxic hepatitis, or impacted calculus in the common bile duct. The values ranged from 11.5 to 26.6 gm. and averaged 17.47 gm. per cent.

The average fat content of the livers of four patients with benign gastrointestinal lesions, the second control group, was 11.82 gm. per cent, and ranged from 6.2 to 17.0 gm. per cent. Although these values are significantly lower than those of the other two groups of patients studied, they still are higher than those reported for normal livers procured at autopsy. These observations would suggest, that either the fat content of the "normal" liver decreases soon after death, or that the lipid content in the livers of patients with benign gastrointestinal lesions is somewhat increased over normal.

TABLE I

The Chemical Composition of Livers of Patients with Cancer of the Gastrointestinal Tract

Patient	Histologic Appearance of the Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent	Total Protein, gm. per cent	"Albumin," gm. per cent	"Globulin," gm. per cent	Serum Protein, gm. per cent
S. B.	Normal	1.3	12.5				
F. B.	Normal	3.6	22.0				
A. B.	Moderate fatty infiltration	1.9	24.0	14.7	3.9	10.8	6.6
H. D.	Normal	1.9	15.9	17.6	6.7	10.9	7.3
P. F.	Normal	1.0	14.8	17.2	5.1	12.1	7.2
T. G.	Normal	1.45	12.1	15.9	5.8	10.1	6.8
S. H.	Normal	1.8	5.3				
A. H.	Normal	2.2	13.0				
W. J.	Normal	2.7	8.55	15.8	5.5	10.3	5.7
M. P.	Normal	1.4	27.1	16.0	8.05	7.97	5.7
L. R.	Normal	4.0	9.5	17.95	4.6	13.35	7.1
L. S.	Normal	2.0	6.9	16.2	5.8	10.4	4.2
J. S.	Normal	5.8	24.1				
J. T.	Normal	8.3	14.8	17.8	3.2	14.6	6.7
J. T.	Moderate fatty infiltration	2.4	29.0				
V. T.	Normal	1.3	22.4	12.9	4.4	8.5	7.4
S. W.	Normal	2.1	35.0	16.8	4.3	12.5	4.6
E. Y.	Normal	7.3	16.9	15.2	4.1	11.1	6.2
Average		2.91	17.43	16.17	5.10	11.05	

TABLE II

The Chemical Composition of Livers of Patients with Considerable Hepatic Dysfunction

Patient	Disease	Histologic Appearance of the Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent	Total Protein, gm. per cent	"Albumin," gm. per cent	"Globulin," gm. per cent	Serum Protein, gm. per cent
W. K.	Impacted calculus in common bile duct	Moderate fatty infiltration	2.5	26.6				
J. R.	Impacted calculus in common bile duct	Normal	1.3	13.3				
B. R.	Toxic hepatitis	Chronic hepatitis	1.6	18.5	14.9	4.9	10.0	5.2
B. L.	Carcinoma of head of pancreas	Chronic hepatitis, peribiliary	5.1	11.5	12.3	5.0	7.3	7.4
Average			2.62	17.47				

TABLE III

The Chemical Composition of Livers of Patients with Benign Gastrointestinal Lesions

Patient	Disease	Histologic Appearance of the Liver	Glycogen, gm. per cent	Total Lipid, gm. per cent	Total Protein, gm. per cent	"Albumin," gm. per cent	"Globulin," gm. per cent	Serum Protein, gm. per cent
T. B.	Duodenal ulcer	Normal	1.3	14.4	11.4	4.7	6.7	6.5
H. N.	Gastric ulcer	Normal	3.6	9.7	14.6	4.6	10.0	5.8
R. G.	Gastric ulcer	Normal	2.8	17.0	15.8	4.4	11.4	6.3
G. L.	Gastritis	Normal	1.4	6.2	16.6	3.9	12.7	5.9
Average			2.28	11.82	14.6	4.40	10.20	

An inverse relationship previously has been noted between the concentrations of fat and of glycogen in the livers of animals,[20] i.e., a high lipid content often is associated with depleted stores of glycogen. Although evidence of this relationship has been noted in most of the individuals included in the present study, several instances were found in which high values of glycogen were associated with abnormally high concentrations of hepatic lipid (table 1).

In previous studies from this hospital, 58 per cent of patients with gastrointestinal cancer have been found to be hypoproteinemic.[2] Moreover, when these individuals were subjected to surgical manipulation, with resultant depletions their serum protein levels fell, often to exceedingly low values.[21] In most instances, the hypoproteinemia was due principally to a decrease of the albumin fraction.

It has been noted that the livers of fasted animals are depleted of proteins with great rapidity,[22] and that when animals are maintained on high protein diets all the protein fractions of their livers increase.[23] These observations,

therefore, suggest that the liver may store proteins, and that those stores might be abnormally reduced in the hypoproteinemic patients with gastrointestinal cancer. The concentrations of proteins in the normal human liver are not known and so not available for purposes of comparison. One may hazard, perhaps, a comparison of the values obtained with those of animal liver tissue. The total protein in the liver of the rat has been found to average 15.5 gm., the "albumin" 2.1 gm., and the "globulin" 13.4 gm. per cent [24]; in the dog liver the average total protein content has been found to be 17.5 gm. per cent.[25]

Of the 18 patients with gastrointestinal cancer the total hepatic protein was measured in 12. The levels ranged from 12.9 to 17.95 and averaged 16.17 gm. per cent. The "albumin" contents averaged 5.10 gm. and varied from 3.2 to 8.05 gm. per cent, and the "globulin" averaged 11.0 and ranged from 8.5 to 14.6 gm. per cent. No apparent correlation was found between the concentrations of protein in the serum and in the livers of the patients studied.

The two control groups gave similar findings. The total protein contents of the livers of two patients with marked hepatic dysfunction were 12.3 and 14.9 gm. per cent; the "albumin" contents 7.3 and 10.05 gm. per cent. The total protein in the livers of four patients with benign gastrointestinal lesions ranged from 11.4 to 16.6 gm. and averaged 14.6 gm. per cent; that of "albumin" ranged from 3.9 to 4.7 gm. and averaged 4.40 gm. per cent; and that of "globulin" varied from 6.7 to 12.7 gm. and averaged 10.20 gm. per cent.

It is interesting to note that the liver apparently does not contain large stores of "albumin." Assuming an average liver weight of 1,500 gm., the average concentration of 4.1 gm. per cent obtained for hepatic "albumin" would amount only to 61.5 gm. of protein, or about one-quarter that of the normal circulating serum albumin. Thus, if the "albumins" of the liver are similar to those of the serum, or are converted readily into that fraction, the store they constitute for serum albumin replacement is a small one.

Discussion

The chemical composition of the liver has been demonstrated to determine the degree of susceptibility or resistance of the organ to various hepatotoxins. For example, there is general agreement that a high concentration of hepatic lipid is pathologic,[15] and that an adequate quantity of hepatic glycogen is protective,[16] but only in the absence of an abnormal content of liver fat.[17] Furthermore, it has been claimed that the administration of high protein diets to animals results in an increased protein content of the liver and affords protection of the organ against several otherwise toxic factors.[26]

Hence, the observation that the livers of patients with gastrointestinal cancer contain abnormally large amounts of fat and probably small amounts of glycogen, becomes of considerable interest. These patients frequently

receive large amounts of narcotics and barbiturates, and undergo considerable surgical manipulation and prolonged anesthesia. These procedures produce, even in the normal liver, a certain amount of transitory insufficiency.[27, 28] It could be expected, then, that patients with gastrointestinal cancer, whose livers are particularly susceptible to damage because of their altered chemical constitution, often would develop postoperatively a considerable degree of hepatic dysfunction.

The mechanisms by which glycogen exerts its protective effect against hepatic damage thus far is not known. However, since the normal metabolism of lipid depends upon the catabolism of carbohydrate, an adequate store of carbohydrate (glycogen) apparently is necessary to insure against an abnormal utilization of fat [29] and its consequent deposition in the liver.[30] Emphasis has been given to the fact that it is not the mere *presence* of glycogen in the liver but rather its *metabolism* which offers protection to the organ against certain toxic compounds.[17, 31]

Many compounds injurious to the liver normally are detoxified by conjugation with glucuronic acid. There now is reason to believe that the synthesis of that acid may depend upon an adequate store of glycogen.[32] It is interesting to note, in this connection, that 55 per cent of patients with gastrointestinal cancer [2] excreted abnormally small amounts of urinary glucuronates.

The mechanisms by which a high content of lipid contributes to development of liver damage probably are better understood. The abnormal deposition of fat in the liver has been shown to compress the sinusoids and consequently induce ischemia in certain portions of the organ.[33] Furthermore, lipid is deposited in the organ without water [34] (in contrast to the deposition of glycogen [35]), so that the available hepatic water must be distributed through a larger tissue volume; the fluid content of the cells consequently may be reduced, and the solution of essential water-soluble constituents decreased. Finally, the increased lipid content of the organ possibly favors the solution and retention in the tissue of many lipid soluble toxins.[36]

At this time no conclusions can be drawn from the values of the hepatic proteins because of a lack of adequate control material. Had a direct correlation been found between the concentrations of protein in the serum and in the livers of the patients studied, then a reasonable explanation for the hypoproteinemia noted so frequently in these individuals might have been a depletion of the hepatic "protein stores." The data obtained could not provide this explanation. The comparatively small amounts of "albumin" found in the livers of even those individuals who had normal serum protein levels suggest that the liver does not constitute a ready store for serum albumin replacement.

The observation that the livers of patients with gastrointestinal cancer contain abnormally large amounts of fat and probably small amounts of

glycogen, suggests a means whereby the impaired hepatic functions of these individuals might be restored to normal. Recent investigations have shown that the administration of certain dietary constituents to experimental animals with livers depleted of glycogen or infiltrated with fat was able to correct their abnormal hepatic chemical composition and ability to withstand the damaging effects of hepatotoxins.[5, 6] Hence, if the hepatic dysfunction in the patient with gastrointestinal cancer was due to the fact that his liver was more susceptible to hepatotoxins because of its abnormal chemical constitution, then the correction of the altered constitution of his liver and its consequent susceptibility to continual damage, might be followed in time by a restoration to a normal functional capacity. Accordingly, attempts have been made to increase the glycogen content and decrease the fatty infiltration in the livers of these patients by the administration of dietary constituents. The results of that study are presented in the subsequent communication.[19]

SUMMARY

1. A high incidence of fatty infiltration and probable glycogen depletion of the liver has been found in patients with gastrointestinal cancer.

2. The possibility is presented that these abnormalities of hepatic chemical constitution make patients with gastrointestinal cancer particularly serious surgical risks.

3. No relationship could be found between the concentration of protein in the serum and in the livers of patients with gastrointestinal cancer.

BIBLIOGRAPHY

1. ABELS, J. C., GORHAM, A. T., PACK, G. T., and RHOADS, C. P.: Metabolic studies in patients with cancer of the gastrointestinal tract. I. Plasma vitamin A levels in patients with malignant neoplastic disease, particularly of the gastrointestinal tract, Jr. Clin. Invest., 1941, xx, 749–764.
2. ABELS, J. C., REKERS, P. E., BINKLEY, G. E., PACK, G. T., and RHOADS, C. P.: Metabolic studies in patients with cancer of the gastrointestinal tract. II. Hepatic dysfunction, ANN. INT. MED., 1942, xvi, 221–240.
3. STEWART, F.: Private communication. This hospital.
4. CATES, H. B.: Relation of liver function to cirrhosis of liver and to alcoholism: comparison of results of liver function tests with degree of organic changes in cirrhosis of liver and with results of such tests in persons with alcoholism without cirrhosis of liver, Arch. Int. Med., 1941, lxvii, 383–398.
5. GREENE, C. H.: Liver and biliary tract, a review for 1941, Arch. Int. Med., 1942, lxix, 691–714.
6. HAWKINS, W. B.: Liver and bile, *in* LUCK, J. M., and HALL, V. E.: Ann. Rev. Physiol., 1941, Ann. Reviews, Inc., Stanford University, California, iii, 259–282.
7. GOOD, C. A., KRAEMER, H., and SOMOGYI, M.: The determination of glycogen, Jr. Biol. Chem., 1933, c, 485–491.
8. NUTTER, P.: Ph. D. Thesis, Rochester School of Medicine, 1938.
9. SHAFFER, P. A., and SOMOGYI, M.: Copper-iodometric reagents for sugar determination, Jr. Biol. Chem., 1933, c, 695–713.

... Peters, J. W., and Hogben, C. G.: The estimation of albumin and ... serum, Jr. Biol. Chem., 1937, cxx, 481–498.

... D., Page, I. H., and Kirk, E.: A manometric micro-method for deter- ... of carbon in organic compounds, Jr. Biol. Chem., 1933, cii, 635–649.

... A. A., Reeves, E. B., and Gottsch, E.: The relationship between the specific ... and protein content in plasma, serum and transudate from dogs, Jr. Biol. ..., cxxii, 167–174.

... P., Rubin, S. H., and Rinzler, S.: The liver lipids in normal human ... in cases of cirrhosis and fatty infiltration of the liver, Jr. Clin. Invest., ...

... H., and Wozasek, O.: Zur Kenntnis des Glykogengehaltes der Leichenleber. ... Veränderungen bei Diabetes mellitus), Ztschr. f. d. ges. exper. Med. 1931, lxxvii, ...

... E., and Partsch, H.: Untersuchungen über den Glykogengehalt der Leber. ... Untersuchungen über den Glykogengehalt der menschlichen Leber im Moment des ... Ztschr. f. Kinderh., 1927, xlv, 78–92.

... D. C., Peterson, S., and Mahbock, W. G.: The glycogen content of the ... Surgery, 1941, x, 716–729.

... S., Vars, H. M., Theobold, E., Schultz, J., and Johnson, J.: The liver ... and blood concentrations following intravenous glucose administration and ... dog and man in the presence of liver damage, Ann. Surg., 1941, cxiv, 1018–

... S., Vars, H. M., and Ravdin, I. S.: The influence of food stuffs upon the ... the liver to injury by chloroform and the probable mechanism of their ... Invest., 1939, xviii, 277–289.

... M., Murphy, H. T., Pack, G. T., and Rhoads, C. P.: Metabolic ... cancer of the gastrointestinal tract. IX. The effects of dietary ... chemical composition of the liver, Ann. Int. Med., 1944, xx, 580–

... L. F.: The deposition of glycogen with water in the livers of ..., cxxvi, 87–101.

... Pack, G. T., and Rhoads, C. P.: Metabolic studies in patients ... gastrointestinal tract. XI. Post-operative hypoproteinemia in ... cancer, Ann. Surg., 1943, lxxvii, 16–20.

... Lewis, H.: Protein loss from liver during a two day fast, Jr. ..., 17–113.

... I. The question of protein storage, Jr. Biol. Chem., 1936, ...

... T., Eberlin, S. C., Halter R., and Rhoads, C. P.: Hepatic ... affected by the administration of dibenzanthracene, Jr. Exper. ...

... Needham, J., and Green, D. E.: Perspectives in ... Cambridge University Press, London.

... Perkins, W. B.: Arsphenamine liver injury modified by diet. ... protective but fat injurious, Am. Jr. Med. Sci., 1940, excix, ...

... G. H.: Chloroform liver injury increases as protein stores ... on metabolism in these dogs, Am. Jr. Med. Sci., 1940, excix, ...

... E. M.: Studies of hepatic function of the Quick hippuric ... surgical states, Arch. Surg., 1938, xxxvii, 443–445.

... der Verfettung, Berlin. klin. Wchnschr., 1904, xli, 587.

... G. C., and Hershey, J. M.: Choline and liver fat in diabetic ..., lxxix, 94–102.

31. Van Creveld, S.: Glycogen disease, Medicine, 1939, xviii, 1–128.
32. Soskin, S., and Hynan, M.: Physiologic basis of intravenous dextrose therapy for diseases of the liver, Arch. Int. Med., 1939, lxiv, 1265–1270.
33. Connor, C. L.: Fatty infiltration of the liver and development of cirrhosis in diabetes and chronic alcoholism, Am. Jr. Path., 1938, xiv, 347–364.
34. MacLachlan, P. L., Hodge, H. C., Bloor, W. R., Welch, E. A., Truax, F. L., and Taylor, J. D.: Lipids of the fasting mouse. II. The fat to water relationship in the liver and the formation of the liver phospholipids, Jr. Biol. Chem., 1942, cxliii, 473–490.
35. McBride, J. J., Guest, M. M., and Scott, E. L.: The storage of the major liver components; emphasizing the relationship of glycogen to water in the liver and the hydration of glycogen, Jr. Biol. Chem., 1941, cxxxix, 943–952.

Science 103:12-13; 1946

Technical Papers

The Lipotropic Properties of Inositol

C. H. Best, C. C. Lucas, J. M. Patterson, and J. H. Ridout

Banting and Best Department of Medical Research
University of Toronto

In the original report (2) of the discovery of the lipotropic effect of inositol and in subsequent publications (3, 4, 7) it was claimed that inositol exerts a specific effect upon the so-called "biotin fatty liver," which was believed to be the same as that produced by a beef-liver fraction. This was particularly interesting, since the "biotin fatty liver," which was stated to be resistant to choline, was supposed to be characterized by a high content of cholesterol. Inositol was reported to be more effective than choline in reducing the level of cholesteryl esters in the lipides of "biotin fatty livers" and also in those resulting from the feeding of cholesterol (7).

Beveridge and Lucas (1), in this laboratory, found that under certain dietary conditions, however, inositol was not more, but distinctly less, active than choline in reducing cholesteryl esters in liver lipides. Further work (to be published shortly) has shown that under all the experimental conditions chosen (21 comparative experiments) choline was at least equally, and usually more, effective than inositol in reducing bound cholesterol. It was invariably more active in reducing total lipides. Similar results were obtained in rats fed on fat-free diets, on diets containing fat, and on diets containing fat and *cholesterol*. When biotin was injected, choline was distinctly more active in reducing total lipides and was at least as effective as inositol in reducing bound sterols. In the prolonged experiments choline was much more effective than inositol in lowering the cholesteryl esters whether or not biotin was given.

No evidence has been obtained in this laboratory to suggest that there is any difference between the fatty liver produced by biotin and that caused by a high fat diet. The ratio of bound sterol to glyceride is the same in the presence of biotin as in its absence. Groups of rats (usually about 12 animals on each diet) injected with biotin (5 γ daily) responded just as well to choline as did those not receiving biotin. The biotin and nonbiotin groups responded to about the same extent to administration of inositol, this response being less than that produced by an equal quantity of dietary choline. The combined effect of choline and inositol is equally pronounced whether biotin is present or absent. In a recent paper, McHenry (5) has retracted certain of his earlier statements concerning (a) the characteristics of the "biotin fatty liver" (*i.e.* its identity with that produced by feeding a certain liver fraction) and (b) the specific lipotropic properties of inositol on this "biotin fatty liver." Our experimental results, which confirm and extend McHenry's latest findings, lead us to advance further along this pathway and to conclude that there is no evidence that the "biotin fatty liver" exists as a unique phenomenon.

McHenry (5) has stated that "it is obvious that inositol is effective in preventing the fatty liver caused by the beef liver fraction, while choline is not." That the liver fraction contains choline[1] has been noted (6), but the fact seems to have been overlooked in explaining the apparently greater relative lipotropic effect of inositol than choline upon the fatty livers so produced. Addition of further choline had only a small effect, as might be expected. Addition of inositol produced the well-known synergistic lipotropic effect. McHenry (5) has stated that "the effect of a combination of choline and inositol is similar to that produced by the same amount of inositol alone." However, the inositol was *not alone* in his experiments, because the liver fraction which was fed contained large amounts of choline. It is obvious that the fatty liver produced by the liver fraction is somewhat resistant to choline, but McHenry's failure to mention the presence of choline in the liver extract has tended to overemphasize the role of inositol under these particular circumstances. It would be interesting to know the level of liver fat which would result from giving a choline-free liver fraction and to compare the relative effects of choline and inositol under such conditions. Experiments along these lines are at present being conducted.

We have conducted many experiments in which diets containing cholesterol were fed over periods from 3 to 16 weeks and have not noted any preferential effect of inositol in lowering either bound sterols or total lipides. In fact, the lipotropic effect of inositol, which was less than that of choline even in short-term "cholesterol" experiments, diminished in relation to that of choline as the experiments were prolonged. In contrast, little, if any, diminution in the choline effect was observed during 16 weeks.

[1] We are using a similar fraction, obtained from the same source, and by the ennea-iodide procedure have found 9 mg. per cc. of free choline and 24 mg. total choline. The latter figure has been confirmed by microbiological assay.

It may also be recorded here that inositol (30 mg. per day) has not, in our experience, been effective in preventing the occurrence of the hemorrhagic kidneys which develop in young rats on diets low in choline and methionine.

References

1. Beveridge, J. M. R., and Lucas, C. C. *J. biol. Chem.*, 1945, **157**, 311.
2. Gavin, E. G., and McHenry, E. W. *J. biol. Chem.*, 1941, **139**, 485.
3. Gavin, E. G., and McHenry, E. W. *J. biol. Chem.*, 1941, **141**, 619.
4. Gavin, E. G., Patterson, J. M., and McHenry, E. W. *J. biol. Chem.*, 1943, **148**, 275.
5. MacFarland, M. L., and McHenry, E. W. *J. biol. Chem.*, 1945, **159**, 605.
6. McHenry, E. W., and Gavin, E. G. *J. biol. Chem.*, 1940, **134**, 683.
7. McHenry, E. W., and Patterson, J. M. *Physiol. Rev.*, 1944, **24**, 128.

 Biochem. J. 40:368-373;1946

The Influence of Biotin upon the Relative Lipotropic Effects of Choline and Inositol

By C. H. BEST, C. C. LUCAS, JEAN M. PATTERSON and JESSIE H. RIDOUT
Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

(*Received* 4 *February* 1946)

It has been reported recently from this department (Beveridge & Lucas, 1945) that choline was more effective than inositol in decreasing the cholesteryl esters in the livers of rats which had been fed on a diet low in the lipotropic factors. This incidental finding was difficult to reconcile with the repeated claims of McHenry and his collaborators (Gavin & McHenry, 1941*a*, *b*; Gavin, Patterson & McHenry, 1943) that 'inositol is effective under conditions unfavourable for the action of choline particularly when large amounts of cholesterol are present in the liver' (McHenry & Patterson, 1944, p. 159). A more extensive comparison of the relative lipotropic effects of choline and inositol was therefore undertaken and the results of a number of studies in which biotin was fed are reported in this paper. No evidence has been secured to support McHenry's contentions (*a*) that biotin produces a fatty liver characterized by a high content of cholesterol, or (*b*) that the fatty liver observed when biotin is fed is 'definitely resistant to the action of choline' (McHenry & Patterson, 1944, p. 136). Recently MacFarland & McHenry (1945) have modified the original statement from their laboratory and state that the fatty liver produced by administering biotin is partially responsive to choline. They now report that both choline and inositol exert an effect upon the cholesterol content of the liver. While our

experimental results are in agreement with this latter observation, they demand a much more extensive revision of McHenry's original interpretation than has as yet been made. Our results lend no support to the idea still prevalent in the literature that biotin interferes, in any way, with the lipotropic action of choline or that inositol has a selective effect upon the fatty liver observed in experiments in which biotin is administered.

An attempt has been made to determine whether the preliminary period of vitamin depletion used by other workers produces an accelerated deposition of liver lipids during the test period as a specific response to the avitaminosis or as a result of the decreased food intake which is inevitable under these conditions.

METHODS

General. For each experiment about 180 young rats (60–120 g.) of the Wistar strain were placed on depletion diets for 3 weeks (see below). At the end of this period the animals were divided into 17 groups with similar weight and sex distribution and one group was killed to determine the condition of the livers. The remaining 16 groups were then given the test diets as described below. Each diet was given to two groups. One group was killed after 1 week and the other after 3 weeks. Throughout the experiment the rats were housed in individual cages with a false bottom of coarse wire screen. Water was available at all times. Weighed amounts of diet were placed each morning in galvanized iron food trays, designed to minimize spilling. The amount left over and the scattered material were weighed daily, from which individual food consumptions could be calculated.

The animals were killed by a blow on the head. Livers were removed immediately and weighed. When small portions were taken for histological examination, allowance was made for the weights removed.

Extraction and analysis of liver fat. The pooled livers were covered with freshly redistilled acetone and disintegrated in a Waring blendor. The tissue brei was transferred quantitatively to large centrifuge pots and acetone added, equal to five times the weight of the livers. After about 1 hr. the mixture was centrifuged and the acetone extract decanted into a large distilling flask. The residue was extracted at least four times with hot absolute ethanol (3 ml./g. of wet liver) under reflux. In the case of very fatty livers as many as seven extractions have been used. The hot extracts, separated by centrifuging, were added to the flask containing the acetone. The extracted tissue residue was transferred quantitatively to a Büchner funnel with hot ethanol. It was sucked as dry as possible, dried further in a current of warm air, transferred to a vacuum desiccator and brought to constant weight over P_2O_5. Completeness of extraction was verified by saponification of the dry tissue residue with hot alkali, acidification and extraction with light petroleum. Usually the fatty acids thus obtained weighed less than 0·1 % of the total lipid extracted.

The combined extracts were taken to dryness *in vacuo* (bath temperature about 45°) under N_2 or CO_2. The fatty residue was dehydrated by adding 25–100 ml. of a benzene-ethanol mixture (4 : 1) and again distilling off the solvent *in vacuo*. This was repeated if necessary. The lipids in the residue were taken up in a number of small portions of light petroleum (b.p. 30–60°). These were centrifuged at high speed, giving a crystal-clear solution of the lipids which was made to volume at 2° and stored in the refrigerator at this temperature.

Samples were removed (in the refrigerator) on which total lipids, phosphorus and cholesterol (free and total) were determined in duplicate. Phosphorus was measured by the method of King (1932) and cholesterol by a slight modification of the Schoenheimer-Sperry procedure (Sperry & Brand, 1943). Phospholipins were calculated by multiplying the phosphorus content by the factor 25·2; the content of bound cholesterol multiplied by 1·68 was assumed to give the weight of cholesteryl esters; the undetermined residue, calculated by difference, has been referred to as glyceride.

Beveridge & Lucas (1945) have discussed briefly the different methods of expressing results of liver-fat determinations. For ease of comparison with values in the literature, total lipid figures are presented in Tables 1 and 2 as percentage of wet liver weight, and also as mg./liver. From these figures and the other data in the tables, the percentage of wet weight or of dry residue weight for any component may be calculated.

Diets (Depletion Period). In Exp. I, during the 3-week depletion period, a fat-free diet of the following percentage composition was fed *ad lib.*: casein 10, sucrose 84, salt mixture no. 185 (McCollum & Simmonds, 1918) 4, celluflour 2, and cod-liver oil concentrate 0·015 (Ayerst, McKenna & Harrison; 50,000 i.u. vitamin D/g., and 200,000 i.u. vitamin A/g.). This diet, lacking all B vitamins, is essentially the same as that used by McHenry and his collaborators (Gavin & McHenry, 1940) during the depletion period common to most of their studies in this field. Celluflour, which has no lipotropic activity, was substituted for the agar used by McHenry. The food consumption of the animals on this deficient diet steadily decreased from almost 10 g. daily at the beginning to about 3–4 g. at the end of the depletion period.

In Exp. II, the same basal diet was fed during the depletion period, but supplements of both choline and inositol were included (20 mg. of each/day in

8 g. of food; choline was given as chloride, 23 mg./day). The following B vitamins were injected daily in 0·5 ml. physiological saline solution: aneurin hydrochloride 25 μg., pyridoxin 20 μg., riboflavin 20 μg., nicotinic acid 100 μg., calcium pantothenate 100 μg. The rats also received daily injections of a liver concentrate stated to contain 5 μg. biotin (liver concentrate stated to contain 20 μg. biotin/ml. (S.M.A. Corporation); each rat received 0·25 ml.) During each week of the depletion period the animals in Exp. II were fed the same average amount of food consumed by the rats of Exp. I during the corresponding week. Thus, in this experiment, during the depletion period, the diet was adequate with respect to vitamins but the caloric intake was limited to correspond to that in Exp. I.

Test period. All diets, freshly prepared each week, were stored in tightly closed tin cans in a refrigerator at about 2°. In Exp. I the basal ration during the test period was the same as that used during the depletion period, but was supplemented by injections of the following B vitamins: aneurin, pyridoxin, riboflavin, nicotinic acid and calcium pantothenate in the amounts described above under Exp. II. The basal ration supplemented with these vitamins is referred to as the control diet. The diets during the test period of Exp. II were the same as the test diets of Exp. I. One half of the rats in each experiment also received 5 μg. crystalline biotin daily by injection, and lipotropic factors were added to the diet as shown in the tables.

During the test periods the animals in each group were 'group pair-fed' with those on the control ration (Diet I), i.e. the average food consumed/rat/day was determined for group 1 of each experiment and this quantity (plus a small amount to allow for scatter) was offered to each rat in groups 2–8 on the following day. The actual food intakes are given in Tables 1 and 2.

Table 1. *Effects of biotin, choline and inositol on rats after a period of depletion of B vitamins*

(Average initial weight of rats was 90 g. (range 62–199 g.))

	Addition to diet					Liver			Composition of liver lipids (mg./liver)					
											Cholesterol			
Group	Substance	Amount (mg./day)	Survivors / Starters	Av. food intake (g./day)	Wt. change (%)	Av. wet wt. (g.)	Av. dry residue weight (g.)	Total lipids (% wet liver wt.)	Total lipids	Phospholipins	Free	Total	Ester (calc. as oleate)	Glyceride
0	None (depletion period)		8/8	5·4	−21	3·64	0·811	8·28	301	134	7·5	11·9	7·5	152
	(*a*) Diet contains B vitamins but no biotin			Test diets fed for 1 week										
1	None	—	9/9	7·0	+15	5·34	1·154	13·74	734	116	8·9	21·7	21·6	588
2	Choline	20	9/9	7·6	25	4·78	1·044	8·65	414	137	9·8	18·7	15·0	252
3	Inositol	20	9/9	7·8	22	4·75	0·978	10·48	499	140	9·8	17·8	13·5	336
4	Choline + inositol	20 + 20	9/9	7·9	24	4·34	0·960	6·80	295	144	8·9	13·9	8·5	134
	(*b*) Diet contains B vitamins plus 5 μg. of biotin													
5	None	—	9/9	7·9	17	5·96	1·058	19·36	1156	119	8·9	29·5	34·7	993
6	Choline	20	9/9	7·6	25	4·51	0·956	8·38	378	137	9·8	17·8	13·5	218
7	Inositol	20	9/9	7·5	19	4·22	0·876	10·70	452	116	8·4	16·1	13·0	315
8	Choline + inositol	20 + 20	9/9	7·5	21	3·96	0·881	6·30	249	130	8·4	11·7	5·6	105
	(*a*) Diet contains B vitamins but no biotin			Test diets fed for 3 weeks										
1	None	—	7/9	8·2	+45	6·02	1·210	15·17	913	158	11·4	36·4	42·1	702
2	Choline	20	8/9	7·9	49	5·33	1·287	7·99	426	134	9·1	20·0	18·4	264
3	Inositol	20	9/9	7·7	36	5·69	1·144	10·09	575	136	10·0	27·8	30·0	399
4	Choline + inositol	20 + 20	9/9	8·1	51	5·36	1·232	5·78	311	150	9·8	13·9	6·9	144
	(*b*) Diet contains B vitamins plus 5 μg. of biotin													
5	None	—	9/9	8·1	43	6·67	1·193	22·95	1530	150	11·5	54·4	74·3	1294
6	Choline	20	8/9	7·9	49	5·10	1·194	7·84	399	139	9·5	19·0	16·0	234
7	Inositol	20	9/9	8·1	43	5·41	1·106	13·46	728	130	10·0	27·2	29·0	559
8	Choline + inositol	20 + 20	9/9	7·9	49	4·86	1·140	6·04	295	133	8·9	12·2	5·7	147

Table 2. *Effects of biotin, choline and inositol on rats after caloric restriction but without vitamin depletion*

(Average initial weight of rats was 76 g. (range 57–101 g.))

Group	Addition to diet: Substance	Addition to diet: Amount (mg./day)	Survivors: Starters	Av. food intake (g./day)	Wt. change (%)	Liver: Av. wet wt. (g.)	Liver: Av. dry residue weight (g.)	Liver: Total lipids (% wet liver wt.)	Composition of liver lipids (mg./liver): Total lipids	Phospholipins	Cholesterol: Free	Cholesterol: Total	Cholesterol: Ester (calc. as oleate)	Glyceride
0	B vitamins, biotin, choline and inositol (see text)		10/10	5·8	−6·4	4·55	1·032	5·09	232	126	8·8	10·5	2·9	94
	Test diets fed for 1 week													
	(*a*) Diet contains B vitamins but no biotin													
1	None	—	9/9	7·5	+13	5·41	1·166	14·28	772	129	10·0	23·3	22·5	610
2	Choline	20	9/9	7·6	17	4·79	1·177	6·60	316	123	9·1	13·9	8·1	176
3	Inositol	20	9/9	7·3	17	5·39	1·127	12·28	662	133	10·6	22·2	19·5	499
4	Choline + inositol	20 + 20	8/8	7·7	20	4·60	1·119	5·40	248	122	8·1	11·9	6·2	112
	(*b*) Diet contains B vitamins plus 5 μg. of biotin													
5	None	—	9/9	7·6	15	5·74	1·203	16·36	940	140	10·3	28·9	31·3	758
6	Choline	20	8/9	7·7	17	4·91	1·140	7·63	374	134	9·4	16·3	11·6	219
7	Inositol	20	9/9	7·8	16	5·12	1·082	12·64	648	126	9·4	21·7	20·7	492
8	Choline + inositol	20 + 20	7/8	7·7	17	4·58	1·070	5·33	244	130	9·0	12·1	5·2	100
	Test diets fed for 3 weeks													
	(*a*) Diet contains B vitamins but no biotin													
1	None	—	9/9	7·8	+29	6·31	1·418	16·99	1050	144	11·5	38·4	45·3	849
2	Choline	20	8/9	7·8	42	5·54	1·132	7·47	419	158	10·6	20·0	15·8	235
3	Inositol	20	9/9	7·8	37	5·47	1·212	8·48	464	144	10·3	24·4	23·8	286
4	Choline + inositol	20 + 20	8/8	7·8	44	5·24	1·206	6·17	324	165	9·8	13·8	6·8	142
	(*b*) Diet contains B vitamins plus 5 μg. of biotin													
5	None	—	9/9	7·3	25	5·98	1·128	17·42	1042	140	11·1	41·7	51·5	839
6	Choline	20	9/9	7·8	40	5·09	1·152	7·42	378	154	10·0	17·2	12·2	202
7	Inositol	20	9/9	7·9	31	5·50	1·146	12·54	690	154	11·1	28·3	29·2	496
8	Choline + inositol	20 + 20	7/8	7·9	39	5·06	1·179	5·73	290	149	9·7	13·6	6·6	125

RESULTS

The results are given in Tables 1 and 2. The data for Fig. 1 are based on those in Table 1.

DISCUSSION

The increased deposition of liver lipids which has been reported (McHenry & Patterson, 1944) to occur following the injection of biotin, has been observed in some but not in all of our experiments. This increase is clearly demonstrated by the data in Table 1 (cf. groups 1 and 5). This effect of biotin is also seen but to a lesser extent (Table 2) when, as in Exp. II, there was no preliminary 3-week period of vitamin depletion. In this second experiment the food intake was, however, restricted during the 3-week preliminary period to correspond to that consumed by the rats in the first experiment when the latter were on the diet lacking the B vitamins. Vitamin depletion during the preliminary period is

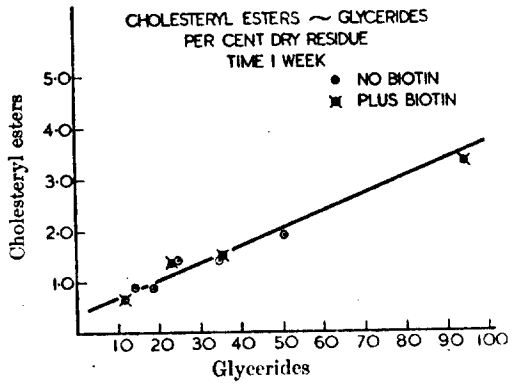


Fig. 1. Relation between cholesteryl esters and glycerides in liver lipids.

thus not necessary to produce a rapid deposition of liver lipids, but a lowered caloric intake may be. We have also encountered conditions under which biotin has little if any effect upon the quantity of lipids deposited in the liver: (*a*) after the preliminary period of depletion of B vitamins, when a moderately large amount of fat (20 % beef dripping) was added to the control diet in the test period, and (*b*) in one experiment when no fat was added to the test diet (i.e. under conditions similar to those of Exp. I), but in which the control group of rats developed particularly fatty livers.

The data presented in Tables 1 and 2 indicate that the fatty liver produced by giving biotin is not characterized by a selective deposition of cholesteryl esters. This finding has been confirmed in other experiments not reported here. Numerous scatter diagrams were prepared to illustrate more clearly the relationship of the levels of cholesteryl esters to those of glycerides in liver lipids of rats on various diets. Because of their essential similarity, only one of these diagrams is presented. In Fig. 1, which is based on data in Table 1, an almost linear increase of cholesteryl esters is seen to occur as the glycerides accumulate. The points lie on the same line whether or not biotin is present. Thus there is no support for the statement that the so-called biotin fatty liver is characterized by an excessive accumulation of bound cholesterol.

The lipotropic effect of choline upon the total liver lipids is invariably greater than is that of inositol, whether biotin is present or not. The data in Tables 1 and 2 show that both glycerides and steryl esters were reduced by choline to essentially the same levels whether or not biotin was given to the rats. Fatty livers produced by injections of 5 μg. crystalline biotin (following a 3-week period of vitamin or caloric depletion) obviously do not show any specific resistance to the action of choline.

Neither the specific lipotropic effect of inositol upon 'biotin fatty livers' nor its specific effect upon cholesteryl esters, originally claimed by McHenry, was noted in any of some twenty experiments. Those in which cholesterol was fed will be reported in a later communication. In short-term experiments (1 week) choline was at least as effective as inositol in reducing the bound cholesterol content of liver lipids. The presence of biotin did not affect this result. In more prolonged experiments (3 weeks) choline was more active than inositol in this respect and again the presence of biotin did not alter the result in any way.

The synergistic effect of a combination of choline and inositol is definitely shown in Tables 1 and 2. Both glycerides and bound sterols are reduced to a significantly lower level by the combination of these two lipotropic factors than by either one alone. Biotin does not interfere with the lipotropic effect of the combination.

It may be mentioned that there is no evidence of any effect of choline, inositol or biotin on the absolute amount of phospholipin or free cholesterol in the livers (Tables 1 and 2). The kidneys of all the rats used in these experiments were analyzed and no effect of these supplements on any component of the lipids was observed.

A number of deductions have been made by McHenry and his collaborators which have been based on the now untenable conclusion that 'choline has no appreciable effect in preventing biotin fatty livers' (Gavin *et al.* 1943, p. 278). Two of these may be mentioned. McHenry & Cornett (1944, p. 9) state that they have found that the addition of biotin to high carbohydrate diets containing pyridoxin and aneurin quickly causes the production of fatty livers containing large amounts of cholesterol but that it fails to do so with protein-rich diets containing the same supplements. On the basis of these results they suggest that the pathway for the synthesis of fat from protein does not proceed by way of carbohydrate. Their evidence does not support this suggestion, since it is probable that the high protein diets provided methionine for the formation of choline, which would presumably inhibit the development of fatty livers under their experimental conditions. McHenry (1941, p. 189) has also speculated that 'Practically all of the existing evidence can be reconciled if we postulate that the development of a fatty liver in the depancreatized dog is due to the positive action of biotin, supplied in the meat of the diet, plus a failure of digestion which prevents the liberation of a lipotropic factor so that it can be absorbed'. The second part of this suggestion does not concern us here. The first part relating to biotin is now pointless in view of the absence of proof of any specific effect of biotin in the production of a particular type of fatty liver in any species. There would appear to be no justification for retaining the term 'biotin fatty liver'.

SUMMARY

1. No evidence has been obtained to support the claims (*a*) that biotin produces a selective deposition of cholesteryl esters in liver, (*b*) that inositol has a specific effect on bound cholesterol, and (*c*) that the fatty liver observed when biotin is administered is particularly resistant to the action of choline.

2. Evidence has been secured that under these experimental conditions, the accumulation of cholesteryl esters in liver bears a remarkably constant relationship to the deposition of glyceride in this organ. The administration of biotin does not affect this relationship.

3. The synergistic effect of choline and inositol on liver lipids has been confirmed.

4. No evidence of any effect of choline, inositol or biotin upon the absolute amount of phospholipin or of free cholesterol in the liver has been obtained in this series of experiments, nor has any effect upon kidney lipids been observed.

5. The absence of a specific effect of biotin on the deposition of liver fat necessitates a revision of certain theories and deductions, two of which have been discussed. It is recommended that the term 'biotin fatty liver' be abandoned.

This work has been financed, in part, by a grant from the Banting Research Foundation.

REFERENCES

Beveridge, J. M. R. & Lucas, C. C. (1945). *J. biol. Chem.* **157**, 311.

Gavin, G. & McHenry, E. W. (1940). *J. biol. Chem.* **132**, 41.

Gavin, G. & McHenry, E. W. (1941*a*). *J. biol. Chem.* **139**, 485.

Gavin, G. & McHenry, E. W. (1941*b*). *J. biol. Chem.* **141**, 619.

Gavin, G., Patterson, J. M. & McHenry, E. W. (1943). *J. biol. Chem.* **148**, 275.

King, E. J. (1932). *Biochem. J.* **26**, 292.

McCollum, E. V. & Simmonds, N. J. (1918). *J. biol. Chem.* **33**, 63.

MacFarland, M. L. & McHenry, E. W. (1945). *J. biol. Chem.* **159**, 605.

McHenry, E. W. (1941). *Biol. Symp.* **5**, 177.

McHenry, E. W. & Cornett, M. L. (1944). *Vitamins and Hormones*, **2**, 1.

McHenry, E. W. & Patterson, J. M. (1944). *Physiol. Rev.* **24**, 128.

Sperry, W. M. & Brand, F. C. (1943). *J. biol. Chem.* **150**, 315.

J. Biol. Chem. 186:317-329;1950

DOSE-RESPONSE CURVES IN THE ESTIMATION OF POTENCY OF LIPOTROPIC AGENTS*

By C. H. BEST, COLIN C. LUCAS, JESSIE H. RIDOUT, AND JEAN M. PATTERSON

(From the Banting and Best Department of Medical Research University of Toronto, Toronto, Canada)

(Received for publication, March 22, 1950)

In the 18 years which have elapsed since the lipotropic activity of choline was discovered, four other naturally occurring substances have been shown to inhibit the deposition or to hasten the removal of excessive amounts of liver fat and to prevent kidney damage. Dimethylpropiothetin, the one most recently discovered (3, 4), was found in the marine alga *Polysiphonia fastigiata*, but is not known to occur in vegetable or animal products commonly used in human or animal rations. The three other substances (betaine, methionine, and inositol) resemble choline in that they are widely distributed and are present in most natural food products. No *systematic* quantitative study of the relative lipotropic activities of these four common dietary components has been reported.

Numerous exploratory studies of lipotropic phenomena by many investigators have provided a broad general picture of the factors involved. Sufficient data are now available to justify a more systematic attempt to determine the relative activity of naturally occurring and synthetic compounds which affect the accumulation of fat in the liver. Since choline appears to be the effective factor when methionine or betaine is given (5, 6), since it is the most active agent of this type, and since inositol exhibits little, if any, significant lipotropic activity in diets containing a normal amount of fat, it would appear logical to use choline as the standard of reference.

Evidence already in the literature indicates that factors which affect appetite and rate of growth influence the choline requirement. For example, Griffith and his colleagues (7, 8) have repeatedly emphasized that dietary supplements which improve the rate of growth should not be considered antilipotropic since there is no evidence for their direct antagonism to choline but there is proof that an increased demand for choline occurs when growth is promoted. A similar situation with respect to the mineral

* Brief accounts of portions of these studies were presented before the American Society of Biological Chemists at Atlantic City, March, 1946, and at Chicago, May, 1947 (1, 2). The expenses of this investigation were defrayed in part by grants from the Banting Research Foundation.

composition of the diet was noticed by Handler (9). When the minerals were inadequate and growth was very slow, sufficient formation of choline occurred from dietary methionine (of the protein) to maintain normal liver lipides. When the minerals were made adequate, growth was resumed. This appropriated more methionine; less was therefore available for conversion to choline and a fatty liver resulted. Beveridge, Lucas, and O'Grady (10, 11) have shown that the state of a diet with respect to essential amino acids may alter or even reverse the findings in certain types of lipotropic studies. Now that such facts are established, the multiple deficiencies in basal diets containing 5 and 10 per cent of casein (often inadequately supplemented with the vitamin B complex), which we and others have sometimes utilized in the past, must be recognized as factors which may affect seriously the outcome of the experiments and interpretation of the data. That environmental conditions (such as extremes of temperature) may also affect the requirement for lipotropic agents is now established (12).

The choice of a basal diet for comparative lipotropic studies offers unique difficulties. The most awkward one is the fact that the essentiality of methionine as an amino acid precludes its complete removal from the diet. Since elimination of methionine was not considered feasible, a basal diet low in methionine, which has been used in previous studies (11), was adopted for this series of experiments. It does not permit any gain in weight, yet suffices for maintenance of rats which have passed the period of most rapid growth (*i.e.*, initial weight beyond 75 gm.). Although this diet supplies slightly less of several other essential amino acids (threonine, tryptophan, and histidine) than the minimal amounts tentatively proposed by Rose *et al.* (13, 14), it seemed satisfactory for the particular investigation contemplated, *viz.*, a study of the lipotropic relationships in a diet sufficing to maintain weight. In a subsequent paper data will be presented showing the effect of increasing increments of growth upon the choline requirement.

EXPERIMENTAL

In the present study, basal diets have been used containing (Series I) no fat, (Series II) moderate quantities of fat (10 per cent beef fat plus 2 per cent corn oil), and (Series III) a larger amount of fat (30 per cent beef fat). These basal diets (in which the methionine content was deliberately restricted) were, of course, low in organic sulfur. Therefore, in one series of experiments (Series IV) a small supplement of amino acid sulfur (as cystine, 0.4 per cent) was added to the diet containing 12 per cent fat.

Choline, betaine, and inositol have been added in varying amounts to the basal diets. The lipotropic agents were usually incorporated at levels of 1, 2, 4, 8, 16, and 32 mg. per 10 gm. of food. Choline was supplied as the desiccated chloride in amounts 1.15 times these values and betaine as the hydrochloride in amounts 1.31 times the values. (The effects of methionine supplements will be reported in a separate paper.)

White rats of the Wistar strain (reared in our colony and weighing usually from 70 to 100 gm.) were housed in individual all-metal cages with a false bottom of coarse wire screen. The test diets were fed for 21 days. The groups (containing ten or more animals each) were comparable with respect to weight and sex distribution within each of the four series of experiments. The rats within each series were "group pair-fed" to minimize effects due to differences in food intake of different groups. For example, in Series I, the rats eating the fat-free basal diet tended to consume less food than did those eating this basal diet supplemented with lipotropic substances. Each rat in all of these groups was therefore offered only the average amount of food eaten per day by the group of rats on this basal diet. The care of the animals and the procedures used for the extraction and analysis of liver lipides have been described previously (15).

Series I. Fat-Free Diet—The basal diet for the experiments of Series I possessed the following percentage composition: casein (Smaco, fat-free, vitamin-free) 8, gelatin (Davis) 12, sucrose 72, salt mixture (16) 5, Cellu flour 2, "vitamin powder" 1, cod liver oil concentrate (Ayerst, McKenna and Harrison, Ltd., Montreal; contains 200,000 i.u. of vitamin A and 50,000 i.u. of vitamin D per gm.) 0.015. The "vitamin powder" consisted of thiamine hydrochloride 500 mg., riboflavin 250 mg., pyridoxine hydrochloride 200 mg., calcium pantothenate 1000 mg., and nicotinic acid 1000 mg., made to 1000 gm. with finely powdered (100 mesh) sucrose. This diet supplies 3.7 kilocalories per gm.

Preliminary work had shown that in rats eating such a ration there was a negligible increase in liver lipides during the 1st week, that a rapid rise occurred during the 2nd week, and that during the 3rd week any further increase was questionable (Fig. 1). There appears to be a tendency for the liver lipides to be slightly higher and more variable in female than in male rats. In all subsequent experiments test diets were fed for 21 days (unless otherwise stated) to be sure that the animals were in a condition of equilibrium with respect to liver fat. The weights of rats on this basal diet remained practically constant during this period. Supplements of choline or betaine produced slight gains in weight, while supplements of inositol did not.

The results of these studies may be presented most concisely in graphical form. Fig. 2 shows the amounts of lipotropic supplements added to the test diets, and the resulting total lipide content of the livers. Fig. 3

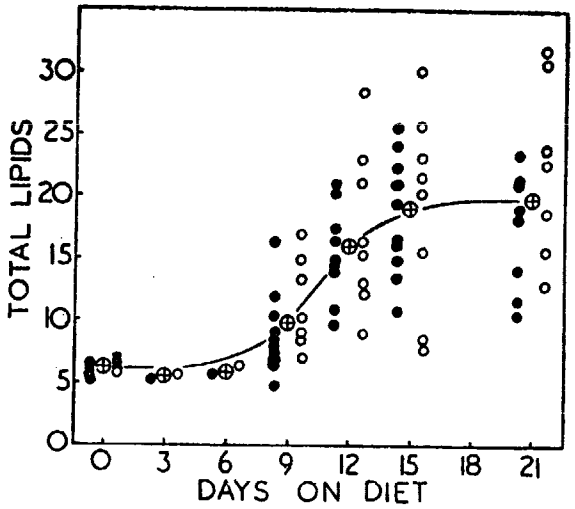


FIG. 1. Rate of development of fatty livers on the fat-free hypolipotropic diet. Total liver lipides expressed as per cent of fresh weight of liver. Individual values for male (●) and female (○) rats (105 ± 15 gm.) are shown with the grand average (⊕). On the 3rd and 6th days the livers were pooled for analysis. Average food consumption about 7.5 gm. per day.

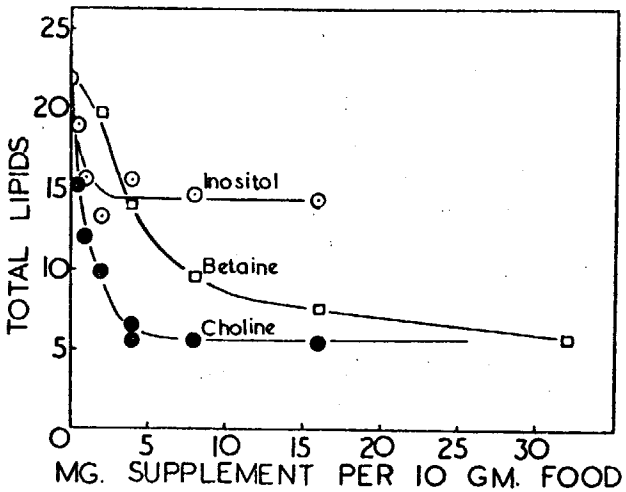


FIG. 2. Dose-response curves showing the effect upon total liver lipides (expressed as per cent of fresh weight of liver) of the lipotropic agents choline (●), betaine (□), and inositol (⊙) in rats consuming the fat-free diet for 21 days. Ten animals (70 to 90 gm.) per group; average food consumption about 8 gm. daily.

illustrates the supplementary lipotropic effect of inositol when added to diets containing choline or betaine. Fig. 4 shows the effect of choline and

inositol, singly and in combination, on the cholesteryl ester content of the liver lipides.

The amount of each of these lipotropic agents required to produce its maximal effect varied considerably. To maintain the liver lipides at a normal level, about 60 mg. of betaine or 8 mg. of choline per 10 gm. of this type of food were required. In contrast, inositol exerted its maximal effect at very low dosage: 2 to 4 mg. per 10 gm. of food reduced the lipides to approximately 15 per cent, and increasing the dosage up to 32 mg. had no additional effect. The supplementary effects of inositol when added to these fat-free diets containing small amounts of choline are apparently real, as shown in Fig. 3, even if the further decrease in the liver lipides is

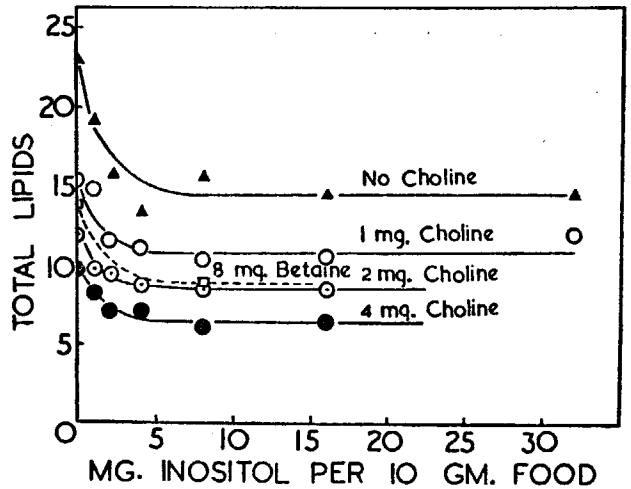


FIG. 3. Dose-response curves illustrating the supplementary lipotropic effects of choline and of inositol in the fat-free diet and of inositol with 8 mg. of betaine per 10 gm. of diet. Total liver lipides expressed as per cent of fresh weight of liver. Rations fed to groups of ten rats each (70 to 90 gm.) for 21 days; average food consumption about 8 gm. daily.

small. Unfortunately these livers were pooled for analysis and hence data are not available for testing the significance of the difference. At maximally effective dosage, choline reduces cholesteryl esters to considerably lower levels than does inositol (Fig. 4), although there is a suggestion that at low dosage levels (below 0.04 per cent in the diet) inositol is somewhat more effective than choline. Inositol exerts a definite but not spectacular supplementary effect upon cholesteryl esters. The ratio of cholesteryl esters to glycerides is not appreciably affected by the presence of inositol in the diets, confirming previous studies (15).

Neither free cholesterol nor phospholipide was altered significantly by the

lipotropic supplements. Since the cholesteryl ester fraction usually accounts for only about 1 to 3 per cent of the total liver lipides, variations in its amount cannot affect the total lipides appreciably. It is obvious, therefore, that choline at all dosage levels exerts its effect mainly upon the glyceride fraction.

Series II. Diets Containing 12 Per Cent Fat—In Series II the diets contained 10 per cent beef fat and 2 per cent corn oil (Mazola) which replaced an equal weight of sucrose in the basal diet used in Series I. The diets of Series II supplied 4.4 kilocalories per gm. The supplements in the test diets and some of the findings are presented in Fig. 5.

Choline is almost as effective in the presence of 12 per cent dietary fat as in the fat-free diet, about 10 mg. per 10 gm. of food giving essentially

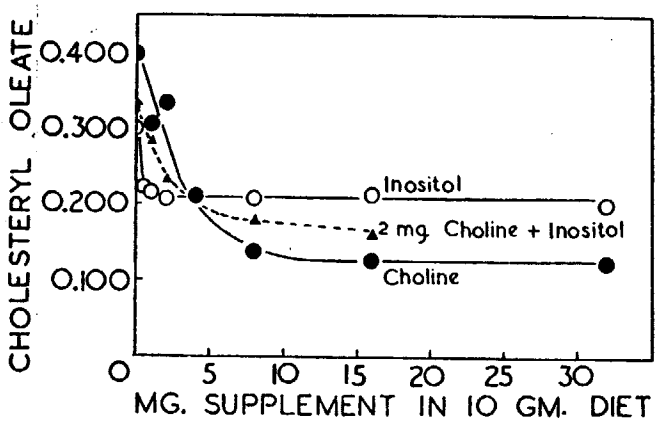


FIG. 4. Effect of lipotropic agents upon cholesteryl esters (expressed as per cent of fresh weight of liver) in liver lipides of rats eating the fat-free diet.

maximal reduction of liver lipides. Owing to the slightly smaller food consumption of these rats, the daily intake of choline required (7 mg.) to produce this effect is essentially the same as in Series I. Inositol failed to exhibit any lipotropic activity whatever in this type of diet.

Series III. Diets Containing 30 Per Cent Fat—These diets, containing 30 per cent of beef fat in place of an equal weight of sucrose in the basal diet of Series I, supplied 5.3 kilocalories per gm. The animals of this series consumed on the average 7 gm. of food per day, corresponding to an average intake of 37 kilocalories as contrasted with 30 and 31 kilocalories in Series I and II, respectively. As might be anticipated, a slightly larger dose of choline was required to give the maximal lipotropic effect with the larger caloric intake. It may be noted (Fig. 5) that the liver lipides did not come down to quite as low a level as they did when less dietary fat was present.

Although betaine was somewhat less effective at lower dosage levels in the diets containing 30 per cent fat than in those free from fat, 64 mg. per 10 gm. of food brought the liver lipides down to normal in both cases. Inositol alone was without effect, and no supplementary action whatever could be detected when it was tested at 1, 2, 4, 8, and 32 mg. levels in diets containing 2 mg. and 8 mg., respectively, of choline.

Series IV. Cystine-Supplemented Diets Containing 12 Per Cent Fat—The basal diet used for Series IV resembled that of Series II but included a supplement of 0.40 per cent cystine. The cystine supplement alone improved the maintenance of weight of the basal group, but little growth

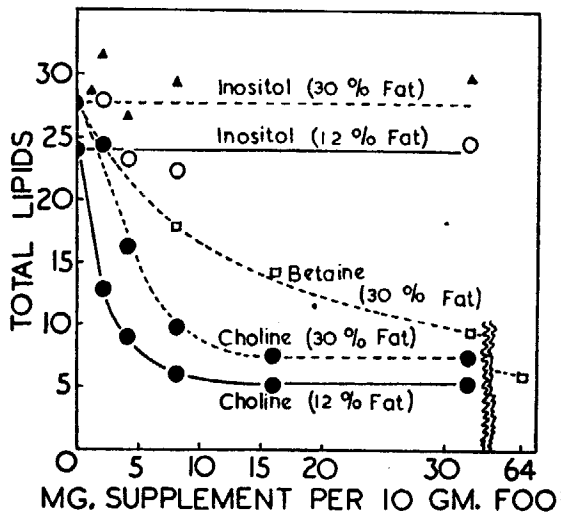


FIG. 5. Dose-response curves showing the total liver lipides (expressed as per cent of fresh weight of liver) resulting from consumption of diets containing 12 and 30 per cent, respectively, of fat with varying amounts of the lipotropic agents. Ten rats (70 to 100 gm.) per group, fed test diets for 21 days; average food consumption about 7 gm. per day.

occurred because other deficiencies (choline first and then possibly threonine and tryptophan) were still limiting factors. The choline supplements that were added to the test diets and some of the findings are shown in Fig. 6. Growth occurred when choline was added, the maximal effect upon weight gain being observed when 8 mg. of choline were present per 10 gm. of food. This increase is possibly due to a sparing influence of choline upon dietary methionine. Choline supplements produced only about one-half as much growth when the cystine supplement was omitted, confirming the suspicion that a deficiency of amino acid sulfur existed in the protein mixture (casein 8 per cent, gelatin 12 per cent of the diet). Inositol

supplements of 2, 4, and 8 mg., respectively, had no beneficial effect upon growth, whether cystine was added or not, and had no effect whatever on the liver lipides (32.6, 34.6, and 32.1 per cent, respectively; basal 33.6 per cent).

The so called "toxic" or "antilipotropic" effect of cystine is seen in the increased deposition of lipides in the livers of the rats on the basal diet. With Griffith (8, 17), we deprecate the application of these terms to cystine, but reserve full discussion of the matter for a future publication. Briefly, the choline requirement is increased by extra growth (which in this case results from an improvement in the organic sulfur content of the ration).

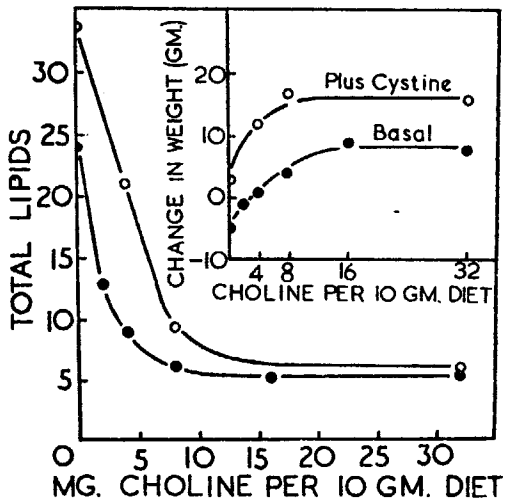


FIG. 6. Lipotropic response of rats (five males and five females per group; 80 to 110 gm.) to different doses of choline in diets with and without supplementary cystine (0.40 per cent). Average food consumption about 7.5 gm. per day. The effects of these supplements on body weight are shown in the insert.

DISCUSSION

Work during the past 25 years on the bioassay of vitamins and on the estimation of the potency of various drugs has shown the necessity of preparing dose-response curves. Extensive investigations of the reliability of such bioassay procedures by chemists, pharmacologists, and statisticians have led to the general recognition of the importance of using only the steep portion of a dose-response curve for making comparisons and have emphasized as another requirement the use of adequate numbers of test organisms. Welch and Welch (18) have already drawn attention to the importance of these considerations in studying lipotropic phenomena. They (19) have reported a dose-response curve for the effect of choline in

mice. In connection with a study of hemorrhagic degeneration of the kidneys in weanling rats, Griffith and Mulford (20) have published a dose-response curve showing the lipotropic effect of choline in young rats on a diet containing 18 per cent casein. No dose-response curves for older rats given any of the lipotropic agents have been found in the literature.[1]

It should be appreciated that the naturally occurring lipotropic agents perform functions in the body which are not necessarily associated with their lipotropic activity. All that is ingested may not be used for lipotropic purposes. Indeed, in the case of methionine, data which it is hoped will soon be published suggest that growth requirements tend to be satisfied before labile methyl groups become available for lipotropic action (through choline formation). The total requirement for any one of the natural lipotropic agents probably differs appreciably from the amount used specifically for lipotropic purposes.

Various estimates of the comparative lipotropic potencies of compounds such as choline, betaine, methionine, inositol, "triethylcholine", arsenocholine, etc., have appeared in the literature (18, 20, 21). It has been found that, in weanling rats on a certain diet, choline possesses 3 times the lipotropic potency of betaine and of methionine (20). These observations have been widely accepted as indicating that methionine methyls are efficiently utilized but that probably only one of the betaine methyls is available for lipotropic action. Several years earlier, Platt (21) had suggested that betaine is only about 30 per cent as effective as choline in preventing deposition of fat in the liver. Realization that the nature of the basal diet, the food intake, and the age and sex of the animal (and possibly other factors yet to be determined) all affect the "choline equivalent" of a lipotropic agent makes it very doubtful that a set of conditions happened upon by chance would give ratios of general physiological significance.

It is, therefore, questionable whether such comparative data as are at present available justify the conclusions that have been drawn as to the relative metabolic availability of methyl groups in different compounds.

Even in the simpler case of merely comparing lipotropic potencies, the curves here presented illustrate the grossly erroneous interpretations that may be made if comparisons are based upon arbitrarily chosen doses of these substances. Data presented elsewhere (22) confirm the statement of Griffith (23, 24) that the lipotropic requirement is related to food consumption, *i.e.*, caloric intake. In the present study, since within any one series the average food intakes of rats in different groups are equal, one may compare lipotropic intakes in terms of the amounts in the diets. For

[1] The application of such curves to the comparison of the lipotropic activity of "triethylcholine" with that of choline is reported in a paper from this laboratory (25).

example, if one found (as we report in Fig. 2) in a fat-free diet that a certain amount of choline, say 0.01 per cent, gave the same percentage of liver fat as did 0.32 per cent inositol, the conclusion might be drawn that choline is 32 times as active as inositol; yet when the dose-response curves are available, it may be seen that 0.04 per cent inositol in a diet produces essentially the same amount of liver fat as did 0.32 per cent. Comparison under these conditions leads to the more nearly correct conclusion that choline possesses 4 times the lipotropic potency of inositol. Even this statement of their relative lipotropic potencies is at best only a half-truth, however, since with this dosage of inositol the liver lipides are left at a moderately high level, about 15 per cent, and more inositol does not exert any further effect. In contrast, the addition of more choline to the diet brings the liver lipides down to normal (about 5 to 6 per cent).

This example illustrates the importance of knowing the characteristics of the dose-response curves of the compounds to be compared. It is now well known that unless the dose-response curves of the substances being compared possess essentially similar characteristics, the ratio of potency of one substance with respect to another will vary with the dosage. In other words, a comparison at any *one* dosage level has no general applicability. For example, in Fig. 2 it may be seen that 4 mg. of betaine give the same liver fat value (19.5 per cent) as does about 0.4 mg. of choline. Under these conditions choline is about 10 times as active as betaine; when 8 mg. of betaine are present the liver fat is reduced to 14.3 per cent, a level which is also produced by about 1.4 mg. of choline, giving a potency ratio of just over 5:1. However, if the comparison is made with 32 mg. of betaine (liver fat about 7.5 per cent), the choline requirement for equal reduction of liver lipides is about 5.5 mg. or a potency ratio of 6:1. Finally, since 64 mg. of betaine per 10 gm. of diet are required to reduce the liver fat to normal and only about 8 to 10 mg. of choline are necessary for this purpose, the ratio here is between 8:1 and 6:1. This point has been labored at considerable length in order to show how these ratios are affected by the conditions under which the potencies are compared and that hence no general statement which is applicable to all dosage levels can be made.

It may be mentioned that some years ago, before the ideas discussed above were appreciated, several attempts to compare lipotropic activity were made in which the *decreases* in liver fat of rats on different test diets were compared. However, it is well known that the quantity of fat deposited on a hypolipotropic basal diet may vary considerably, for reasons not yet elucidated. *Mean values* ranging from 20 to 30 per cent of wet liver weight have been encountered when similar (small) groups of rats were fed apparently identical basal diets for the same test period at different seasons. Added choline (say 0.1 to 0.2 per cent) reduces the liver fat to a fairly constant level (about 5 to 8 per cent). This lower level seems to be much more reproducible than is the high level on the basal diet. Estimates of relative potency, based on decreases measured from the *variable* high level, lack reproducibility because of this variability and are, moreover, meaningless from the point of view of dose-response curves.

Experimental studies and clinical observations during recent years have promoted the lipotropic agents to a prominent position among the dietary components. With an increasing realization of their essential nature, the relative potencies of the various substances possessing lipotropic activity assume greater practical importance. The complex biochemical and physiological interrelationships of these compounds, particularly in the rat and in chicks, have been partially elucidated (25–27). Studies *in vitro* by Borsook and Dubnoff (28, 29) have shown that the enzymes responsible for the various transmethylation reactions are highly specific and that the distribution of the individual enzymes varies from species to species (30).

Raymond and Treadwell (31) have mentioned the desirability of adopting standardized conditions for the comparison of lipotropic activities. Interpretation of the significance of the data they present is impossible because there is no way of knowing whether the one dosage level used in their tests is on the steep part or on the flat portion of the respective dose-response curves. This defect is common to most papers on lipotropic phenomena. Recognition of this weakness is indicated in the recent paper by Welch (27) on the relation of molecular structure of choline analogues and homologues to their protective ability against hemorrhagic renal lesions of dietary origin. Welch reports the effects of eight dosage levels of choline and of two to six levels of the other compounds. There are, of course, commendable features in the proposal to use standardized conditions for comparisons, provided these are chosen with sufficient forethought and with the realization that no one set of conditions can supply a complete answer.

Recent reports (32–34) indicate that vitamin B_{12} and folic acid exert a sparing action upon the choline requirement. The data presented in this paper show the necessity of having dose-response curves available in order to estimate accurately the requirement for each of the lipotropic agents under different dietary and environmental conditions.

SUMMARY

1. Evidence has been presented showing the necessity of preparing dose-response curves for the accurate estimation of lipotropic requirements under stated dietary and environmental conditions.

2. Dose-response curves are given showing the lipotropic effects of choline, betaine, and inositol in fat-free diets and in diets containing fat.

3. Choline exerts a strong, and betaine a moderate, lipotropic effect under all dietary conditions studied.

4. Inositol exhibits a relatively limited lipotropic effect in fat-free diets but shows no activity when the diet contains fat. The supplementary lipotropic action of inositol (with choline or betaine) in fat-free diets has been confirmed but this supplementary effect is not observed in diets containing fat.

5. The impossibility of making a general statement concerning the activity of any one of the naturally occurring lipotropic substances with respect to any other is obvious from the marked dissimilarity of the dose-response curves.

6. Previously reported lipotropic ratios of 3:1 for betaine and methionine with respect to choline are of questionable significance since the comparisons were made under conditions which have now been shown to be unsatisfactory for establishing such relationships.

BIBLIOGRAPHY

1. Lucas, C. C., Best, C. H., Ridout, J. H., and Patterson, J. M., *Federation Proc.*, **5**, 145 (1946).
2. Lucas, C. C., Ridout, J. H., Patterson, J. M., and Best, C. H., *Federation Proc.*, **6**, 273 (1947).
3. Challenger, F., and Simpson, M. I., *Biochem. J.*, **41**, p. xl (1947).
4. Maw, G. A., and du Vigneaud, V., *J. Biol. Chem.*, **176**, 1037 (1948).
5. Welch, A. D., and Landau, R. L., *J. Biol. Chem.*, **144**, 581 (1942).
6. McArthur, C. S., Lucas, C. C., and Best, C. H., *Biochem. J.*, **41**, 612 (1947).
7. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **131**, 567 (1939).
8. Griffith, W. H., and Mulford, D. J., *J. Nutr.*, **21**, 633 (1941).
9. Handler, P., *J. Biol. Chem.*, **149**, 291 (1943).
10. Beveridge, J. M. R., Lucas, C. C., and O'Grady, M. K., *J. Biol. Chem.*, **154**, 9 (1944).
11. Beveridge, J. M. R., Lucas, C. C., and O'Grady, M. K., *J. Biol. Chem.*, **160**, 505 (1945).
12. Sellers, E. A., and You, R. W., *Science*, **110**, 713 (1949).
13. Rose, W. C., *Science*, **86**, 298 (1937).
14. Rose, W. C., Oesterling, M. J., and Womack, M., *J. Biol. Chem.*, **176**, 753 (1948).
15. Best, C. H., Lucas, C. C., Patterson, J. M., and Ridout, J. H., *Biochem. J.*, **40**, 368 (1946).
16. Beveridge, J. M. R., and Lucas, C. C., *J. Biol. Chem.*, **157**, 311 (1945).
17. Griffith, W. H., *Biol. Symposia*, **5**, 193 (1941).
18. Welch, A. D., and Welch, M. S., *Proc. Soc. Exp. Biol. and Med.*, **39**, 7 (1938).
19. Welch, M. S., and Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **39**, 5 (1938).
20. Griffith, W. H., and Mulford, D. J., *J. Am. Chem. Soc.*, **63**, 929 (1941).
21. Platt, A. P., *Biochem. J.*, **33**, 505 (1939).
22. Best, C. H., Hartroft, W. S., Lucas, C. C., and Ridout, J. H., *Brit. Med. J.*, **2**, 1001 (1949).
23. Mulford, D. J., and Griffith, W. H., *J. Nutr.*, **23**, 91 (1942).

24. Griffith, W. H., in Evans, E. A., Jr., The biological action of the vitamins, Chicago (1942).
25. Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 373 (1942).
26. Jukes, T. H., *Ann. Rev. Biochem.*, **16**, 193 (1947).
27. Welch, A. D., *J. Nutr.*, **40**, 113 (1950).
28. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **171**, 363 (1947).
29. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **176**, 789 (1948).
30. Dubnoff, J. W., *Arch. Biochem.*, **24**, 251 (1949).
31. Raymond, M. J., and Treadwell, C. R., *Proc. Soc. Exp. Biol. and Med.*, **70**, 43 (1949).
32. Schaefer, A. E., Salmon, W. D., and Strength, D. R., *Proc. Soc. Exp. Biol. and Med.*, **71**, 193 (1949).
33. Drill, V. A., and McCormick, H. M., *Proc. Soc. Exp. Biol. and Med.*, **72**, 388 (1949).
34. Schaefer, A. E., Salmon, W. D., Strength, D. R., and Copeland, D. H., *J. Nutr.*, **40**, 95 (1950).
35. McArthur, C. S., and Lucas, C. C., *Biochem. J.*, **46**, 226 (1950).

J. Biol. Chem. 157:311-321; 1945

THE EFFECT OF DIETARY FAT ON THE LIPOTROPIC ACTION OF INOSITOL

By J. M. R. BEVERIDGE and COLIN C. LUCAS

(From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada)

(Received for publication, October 13, 1944)

The possibility that choline exerts lipotropic action through its introduction into newly formed lecithin molecules is an attractive hypothesis for which there exists some supporting evidence (1–3). Inositol, which is also a constituent of phospholipid (4, 5), may possibly exert its effect in the same way. McHenry and Patterson (6) have already suggested this possibility. If these two substances actually do lower liver fat by virtue of their incorporation into phospholipid molecules, then essential fatty acids, which also constitute integral parts of certain phospholipids, might similarly be required before choline and inositol could exert their effects, or be necessary to produce maximal lipotropic action.

About the time that experimental work was started to test these points, Engel published a paper (7) in which he mentioned the relation of the essential fatty acids to the lipotropic action of choline. His motives for carrying out this particular investigation differed from those which initiated the present study. Engel had found that pyridoxine is necessary for choline to exert its full lipotropic effect, and because of the complementary relationship of pyridoxine and essential fatty acids in the cure of rat acrodynia (8, 9) he was prompted to examine the possible influence of essential fatty acids on the lipotropic action of choline. Engel fed 0.1 ml. of corn oil to each test rat daily. This supplement did not alter the lipotropic effect of choline in short term experiments (3 weeks), but did augment the latter's action in an experiment of 8 weeks duration. In a recent publication (10) by one of us, data obtained from a small series of animals supported this finding of Engel's. Unexpectedly, it was also discovered that corn oil (Mazola brand), which was fed with the object of supplying essential fatty acids, instead of augmenting the lipotropic action of inositol actually obliterated it. The present paper confirms this observation and reports more detailed analytical data for liver fat from rats on a number of diets similar to those utilized in the preliminary work reported by Beveridge (10).

EXPERIMENTAL

Young rats of the Wistar strain were used for the feeding experiments which ran 8 weeks. The fat-free basal diet consisted of the following

components:[1] casein (fat- and vitamin-free, Smaco brand) 8 per cent, gelatin 12 per cent, sucrose 73 per cent, salt mixture[2] 5 per cent, agar 2 per cent, cod liver oil concentrate (500,000 I.U. of vitamin A per gm. and 50,000 I.U. of vitamin D per gm. (Ayerst, McKenna and Harrison)) 0.015 per cent. The casein and gelatin were extracted with hot alcohol-ether.

The B vitamins were administered daily by subcutaneous injection in 0.5 ml. of 0.9 per cent saline: thiamine hydrochloride 50 γ, riboflavin 25 γ, pyridoxine 20 γ, Ca pantothenate 100 γ, nicotinic acid 100 γ. The supplements, which were added at the expense of the sucrose, were given as described in the text.

Series A—This series was planned to test the effects of essential fatty acids (supplied by feeding corn oil) on the lipotropic action of choline and inositol. Twenty young rats (23 to 35 days old) were used for each group and litters were divided as evenly as possible among the different groups. The groups were also balanced with respect to weight and sex.

The diets were made up fresh every 10 to 14 days. The fatty ingredients were incorporated by spraying them, in dilute solution in acetone, over thin layers of the other components, which were then thoroughly mixed with a spatula until all odor of the solvent had disappeared. Further mixing was done by crumbling the mass by hand. The diets were stored at 4° in tightly closed containers. The animals were fed *ad libitum*.

With the exception of Groups 6B and 7B individual liver fat was determined in the usual way by saponification, acidification, and extraction of the fatty acids[3] with petroleum ether. The livers of Groups 6B and 7B were pooled and the fat was extracted essentially according to the methods described by Artom and Fishman (12). Iodine numbers were determined according to the method of Rosenmund and Kuhnhenn as modified by Yasuda (13). Phospholipid was estimated by multiplying the total phosphorus of the lipid fraction by the Artom and Fishman (12) factor 22.7; cholesterol was determined gravimetrically as the digitonide. Details of the supplements and the analytical findings are given in Tables I and II.

Series B—Since the results of Series A had shown that, under the conditions used, the inclusion of corn oil obliterated the lipotropic action of inositol, the effect of a saturated fat (free of essential fatty acids) was also determined. At the same time the effect of this fat was compared with that of corn oil on the lipotropic action of choline. In order to avoid any complicating factors due to differences in food intake, all the groups were

[1] The composition of the basal diet, as described in the preliminary report (10), is confused by a typographical error. The words "12 per cent casein" should be deleted.

[2] The salt mixture used was one based on the McCollum Salt Mixture 185 (11) modified according to the best available data concerning the mineral requirements for growing rats. The composition, in gm. per 100 gm. of salts, is as follows: Ca lactate ($5H_2O$) 35.15, $CaCO_3$ 5.28, $Ca(H_2PO_4)_2 \cdot H_2O$ 14.60, K_2HPO_4 6.45, $NaH_2PO_4 \cdot H_2O$ 18.76, NaCl 9.34, $MgSO_4$(anhydrous) 7.19, ferric citrate ($3H_2O$) 3.19, $MnSO_4 \cdot 2H_2O$ 0.33, $ZnSO_4 \cdot 7H_2O$ 0.035, $CuSO_4 \cdot 5H_2O$ 0.039, KI 0.00039.

[3] The material extracted, which is usually referred to as "crude liver fatty acids," contains also the unsaponifiable matter.

TABLE I

Effect of Corn Oil on Lipotropic Action of Choline and Inositol, Series A

Groups of twenty rats, average weight 62 gm., were fed experimental diet for 5 weeks.

Group No.	Supplement	No. of survivors	Change in weight of survivors	Food intake	Fatty acids as per cent of wet liver weight Average
	per cent		*per cent*	*gm.*	
1	None (basal)	14	+24	7.8	23.4
2	Choline chloride (0.5)	16	+71	9.9	6.4
3	Inositol (0.3)	13	+20	8.6	13.3
4	Corn oil (1.0)	15	+25	7.9	25.3
5	" " (1.0) + inositol (0.3)	11	+33	8.7	27.2
6A	" " (1.0) + choline chloride (0.5)	17	+112	9.6	4.7
7A	" " (1.0) + choline chloride (0.5) + inositol (0.3)	19	+105	9.8	3.8
6B	Same as Group 6A	17	+94	8.8	4.5*
7B	" " " 7A	16	+99	9.7	3.6*

* Total lipids extracted from the pooled livers by alcohol and alcohol-ether gave values for Group 6B of 6.3 and for Group 7B of 4.6 per cent wet liver weight. Aliquots of these extracts were used for determination of total fatty acids in the usual way by saponification, acidification, and extraction with petroleum ether.

TABLE II

Composition of Fats from Pooled Livers, Series A

The values are expressed as per cent of fat-free, dry liver tissue. The per cent liver residue weight of original wet weight was 22.15 in Group 6B and 23.95 in Group 7B.

Group No.	Total lipid*	Phospholipid†	Cholesterol		Steryl ester‡ calculated as oleate	Glyceride§
			Free	Esterified		
6B	24.8	13.30	0.66	0.37	0.62	10.2
7B	19.2	11.90	0.63	0.37	0.62	6.0

* The iodine number of Group 6B was 81.3 and of Group 7B was 92.1.
† Calculated from P in total lipid (chloroform-soluble P × 22.7).
‡ Esterified cholesterol × 1.68 assumed to give the weight of cholesteryl esters.
§ By difference.

paired fed on Group 4 ("non-essential fat" supplement). The supplements used in this experiment and their effects upon the rats are shown in Table III and the proximate liver analyses are given in Table IV.

The livers, after being weighed and pooled, were emulsified in a Waring blendor with 5 volumes of acetone. Small aliquots were taken for total crude fatty acids, which were determined in the usual way. The remainder of the acetone suspension was made up to about 10 volumes and allowed to

TABLE III

Effect upon Young Rats of Diets Used, Series B

Groups of twenty rats were fed experimental diets for 8 weeks; the food intake was 7.9 gm. per rat per day.

Group No.	Supplements	Average initial weight	Survivors	Average initial weight of survivors	Average change in weight of survivors	Average wet liver weight
	per cent	*gm.*		*gm.*	*per cent*	*gm.*
	None (basal)	68.8	10	75.1	+8	5.10
1	Corn oil (1.0)	70.4	16	77.8	+31	7.52
2	Inositol (0.3)	70.2	13	82.5	+10	5.15
3	Corn oil (1.0) + inositol (0.3)	69.9	15	78.5	+20	7.15
4	Non-essential fat* (1.0)	69.3	12	79.2	+16	6.10
5	" " (1.0) + inositol (0.3)	72.5	13	72.9	+27	4.46
6	Corn oil (1.0) + choline chloride (0.5)	72.4	20	72.4	+36	3.56
7	Non-essential fat (1.0) + choline chloride (0.5)	69.1	18	65.1	+41	3.23

* This material, isolated from beef dripping by repeated fractional crystallization from acetone, had an iodine number of only 5.0. Even if all the unsaturation were due to linoleic acid, the quantity supplied in the diet would amount to only about 2 mg. per rat per day. However, this possibility is most unlikely. In the first place, published analyses of beef dripping show that linoleic acid accounts for only a comparatively small part of the unsaturation of this fat (oleic acid 40 to 50 per cent, linoleic acid 2 to 3 per cent of the fat). Further, since the more unsaturated fats would be preferentially left behind in the fractionation, the small amount of unsaturated glycerides remaining in the fraction is almost certainly due to oleic acid. The melting point of the isolated fraction, which will be referred to as "non-essential fat," was 50–53°.

stand for about half an hour. The acetone dehydrated the liver tissue and took up a portion of the liver fat. The mixture was centrifuged, the supernatant was filtered, and the filtrate was evaporated to dryness *in vacuo* under a stream of nitrogen. After dehydration of the material left in the flask, by addition of absolute alcohol and evaporation *in vacuo*, the fat in the residue was extracted with petroleum ether. The dehydrated and partially defatted liver tissue was extracted in Pyrex centrifuge pots (250 ml.) five times by refluxing for about 30 minutes each time with 3

volumes of absolute alcohol. The alcoholic extracts were evaporated as above and the petroleum ether extract of the residue so obtained was combined with that from the acetone extract. The water-free, fat-free liver tissue was dried *in vacuo* over P_2O_5 and weighed. Saponification of this liver residue revealed that the process used for extraction of the fat was more than 99.5 per cent efficient. Cholesterol was determined by the Schoenheimer-Sperry method (14). Phospholipid was estimated by (*a*) a calculation based on the total phosphorus in the petroleum ether extract by using the factor 25.2 (assuming 93 per cent monoamino phosphatide and 7 per cent diamino phosphatide); (*b*) a calculation based on the phosphorus content of the total crude phospholipid precipitated by acetone and $MgCl_2$.

TABLE IV

Effect of Dietary Fat on Lipotropic Action of Choline and Inositol, Series B

Groups of twenty rats, average weight 70 gm., were fed experimental diets for 8 weeks; the food intake was 7.9 gm. per rat per day.

Group No.	Fatty acids		Total lipid		Unextracted fat	Water	Dry tissue
	per cent wet liver	*per cent dry, fat-free liver*	*per cent wet liver*	*per cent dry, fat-free liver*	*per cent of fat extracted*	*per cent wet liver*	*per cent wet liver*
	30.4	200	31.2	206	0.12	53.6	15.2
1	31.0	241	32.4	253	0.14	54.7	12.9
2	20.0	132	23.5	157	0.26	61.3	15.0
3	28.0	191	33.8	231	0.09	51.5	14.7
4	25.7	173	28.4	191	0.06	56.7	14.9
5	21.0	119	25.1	143	0.11	57.3	17.6
6	5.6	24	8.0	35	0.47	68.7	23.3
7	4.3	19	7.1	31	0.29	69.7	23.2

This precipitation was carried out in 250 ml. centrifuge pots by taking about 50 mg. of liver fat, dissolving this in 5 ml. of petroleum ether, and adding 85 ml. of anhydrous acetone. According to the recommendation of Sinclair and Dolan (15), the precipitation was completed by adding 0.25 ml. of a saturated ethanolic solution of magnesium chloride. After being thoroughly mixed, the material was left in the refrigerator for about an hour. The mixture was centrifuged and the precipitate was washed with 25 ml., 15 ml., and 10 ml., respectively, of acetone. Phosphorus in the precipitate was determined by the method of King (16).

Iodine numbers on the unfractionated fats were determined in the usual way (13).

DISCUSSION

There has been no unanimity among investigators working on lipotropic substances as to the best method of expressing analytical data derived

from lipid determinations on livers of markedly variable fat content. Results have been most commonly expressed as a per cent of the wet weight of liver. The variability of water content of the liver with age and with the degree of infiltration of fat may not only conceal changes which have occurred in the absolute amounts of the lipids present but may also lead to quite erroneous conclusions. Beeston, Channon, and Wilkinson (17) have advocated expressing results as gm. of lipid per 100 gm. rat; *i.e.*, liver fat as a percentage of final body weight. Since the addition or withdrawal of one nutrient often may have a marked effect on body weight without a corresponding effect on the liver, the objection to this method of expressing data is obvious. The use of the initial body weight, while not ideal, avoids

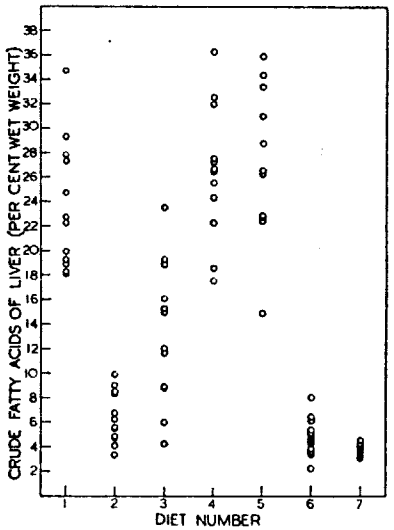


FIG. 1. Individual liver fat values, obtained on diets in Series A

some of the difficulties. Some investigators (*e.g.*, Artom and Fishman (12)) have based their results on the *fat-free* tissue and others (*e.g.*, Engel (7)) on the *moisture-free* tissue. While the two latter methods constitute an improvement, they still leave something to be desired, since in each case insufficient cognizance is taken of one large variable. The present authors believe that liver fat should be expressed as a percentage of the weight of the water-free, fat-free liver since the latter, in the absence of extensive cirrhosis, is a fairly true measure of the relative amounts of active tissue. The data obtained in the present study are therefore so expressed, unless otherwise stated.

The distribution of the individual values for total crude liver fatty acids of Series A is shown in Fig. 1 and the summarized data are presented in

Table I. The data in Table I reveal a curious phenomenon for which no explanation can yet be advanced. On a fat-free diet the lipotropic effect of inositol was definitely demonstrated (Group 3) but the addition of corn oil (Mazola) to the diet completely prevented this action (Group 5). The observation was confirmed in Series B (Table IV (compare Groups 2 and 3)). The addition of an identical amount of a solid fat (iodine No. 5), which may be assumed for all practical purposes to be devoid of essential fatty acids, had no such nullifying effect upon the lipotropic action of inositol (Table IV (compare Groups 2 and 5)). Obviously some material in the Mazola modifies the action of inositol. Since glycerides of the essential fatty acids are major components of corn oil, the possibility that these substances are the agents responsible for the effect is a likely one, although this point has not yet been definitely established.

Engel's report (7) that corn oil augments the lipotropic action of choline appears to be supported by the data in Table I (compare Group 2 with Groups 6A and 6B). Differences in the average values are not of large magnitude but the fact that fifteen out of seventeen individual values in Group 6A are below the average value for Group 2 (see Fig. 1), coupled with the good agreement between the results in Groups 6A and 6B, is evidence that the differences are significant. The data in Table I (compare Group 6A with Group 7A and Group 6B with Group 7B) also appear to confirm Engel's finding that inositol brings about a further lowering of liver fat when added to diets containing an adequate amount of choline, for all nineteen individual values in Group 7A are below the average value for Group 6A. Unequivocal proof of the reality of the supplementary lipotropic effects of corn oil and inositol in diets containing adequate choline would require much larger groups of animals than were utilized in the present studies. However, such effects as were observed at the high levels of choline used in these experiments, although small, were in the direction expected from Engel's work.

Fractionation of the lipids from Groups 6B and 7B of Series A revealed that the reduction of liver fat brought about by the addition of inositol to a diet containing an adequate amount of choline is due mainly to a decrease in neutral fat (Table II).

Although in Series B the percentage of phospholipid in the different fats varied widely (6.9 to 60.6 per cent, Table V), the phospholipid levels calculated on the weight of dry, fat-free liver tissue remained practically constant in the livers of the different groups (Table VI), despite the fact that a number of the diets contained neither choline nor inositol and some were deficient in essential fatty acids. The fact that the total amount of phospholipid was relatively unchanged whether choline was present or not warrants further examination of the phospholipid fractions from the animals

TABLE V

Analytical Data for Fats from Pooled Livers, Series B

The values are expressed as per cent of total lipid.

Group No.	Phospholipid*†	Cholesterol		Steryl ester‡	Glyceride§	Iodine No. of total lipid
		Free	Esterified			
Basal	7.5	0.66	2.27	3.82	88.0	70.2
1	7.1	0.55	1.42	2.39	90.0	82.8
2	10.8	0.69	2.04	3.44	85.1	73.4
3	6.9	0.54	1.43	2.41	90.2	83.0
4	8.8	0.67	1.72	2.90	87.6	71.7
5	12.3	0.82	1.43	2.41	84.5	70.2
6	47.1	2.70	0.85	1.43	48.8	76.1
7	60.6	3.31	1.76	2.96	33.1	68.8

* Calculated from the phosphorus of total lipid (petroleum-soluble P × 25.2).

† The phospholipid figures were those obtained by the method referred to as (*a*) in the text; values by method (*b*) were about 13 per cent lower. However, the values by method (*a*) agreed very closely (usually within 5 per cent) with those calculated from the weight of fatty acids derived from the phospholipid precipitated by the method of Sinclair and Dolan (15). In this latter case, aliquots of the petroleum ether solutions of the total lipids were taken to contain about equal quantities of petroleum-soluble phosphorus. These were evaporated to dryness, taken up in 10 ml. of light petroleum, and treated with 175 ml. of anhydrous acetone. Precipitation was completed by adding 0.45 ml. of ethanolic magnesium chloride solution. The washed precipitates were saponified with 5 ml. of 0.4 N alcoholic KOH, acidified, and extracted with light petroleum. The weight of fatty acids × 1.48 (see Artom and Fishman (12)) was assumed to give the phospholipid in the aliquot.

‡ The esterified cholesterol × 1.68 is assumed to give the cholesteryl ester.

§ By difference.

TABLE VI

Nature and Amount of Liver Lipids, Series B

The values are expressed as per cent of fat-free, dry liver tissue.

Group No.	Phospholipid (P × 25.2)	Cholesterol		Glyceride
		Free	Esters*	
Basal	15.4	1.36	7.84	181
1	18.0	1.39	6.02	228
2	16.9	1.08	5.37	134
3	15.8	1.24	5.55	209
4	16.8	1.28	5.53	167
5	17.6	1.17	3.44	121
6	16.2	0.93	0.49	16.9
7	18.7	1.02	0.90	10.2

on the different diets to determine what changes, if any, have taken place in the choline-containing lipids.

The amount of free cholesterol remained practically constant in all the groups but marked differences in the cholesteryl esters were observed. The average values, expressed as absolute weight of esterified cholesterol per liver, were as follows: on the basal diet 36.1 mg., on Diets 1 to 7, 34.4, 21.7, 34.6, 29.5, 16.0, 2.4, and 4.0 mg., respectively. The high value found for cholesteryl esters on the basal diet is worthy of note. In view of the prominent lipotropic rôle which has been assigned to inositol in controlling cholesterol levels in the liver, it was surprising to find that on the diets used in these studies choline had a much more marked effect than inositol in reducing cholesteryl esters (Table VI, compare Groups 3 and 6). Similar observations were made 10 years ago when Best *et al.* (18) noted that, in rats fed a grain diet, addition of choline caused an appreciable decrease (33 per cent) in the glyceride content of the liver, but a much larger percentage decrease (78 per cent) in the steryl esters. When 40 per cent beef dripping was incorporated into the grain diet, Best *et al.* found that the percentage decrease in steryl esters occasioned by adding choline was even greater (87 per cent). A similar but less marked decrease (59 per cent) in steryl esters was caused by choline in grain diets containing 20 per cent of Crisco and 2 per cent of cholesterol. Inositol and other lipotropic factors were undoubtedly present but were constant in the diets under comparison.

When inositol was added to a diet containing the "non-essential fat," a definite lowering in the cholesteryl esters took place (compare Groups 4 and 5, Table VI), but when inositol was added to a diet containing corn oil and choline, no change in steryl esters was observed (Table II), although a 41 per cent decrease in glycerides was produced. The whole question of the lipotropic action of inositol and the effect of the nature of the basal diet on its action merits further study.

Corn oil plus choline gave the lowest cholesteryl esters resulting from any of the diets used in these experiments (Group 6, Tables V and VI).

The proportion of glyceride in the fats (Table V) shows very wide fluctuations. In general, the variations in the other lipid components appear to be affected to a greater or lesser degree by simple dilution with accumulating glycerides. While the principal action of choline upon the *total amount* of liver lipids as revealed by these studies is to reduce the glycerides, a fact already noted by other workers using different basal diets and different supplements, a similar (and in some cases even greater) proportional reduction in steryl esters is brought about by this lipotropic agent.

The iodine numbers (Table V) of the fats fell into the order expected of them, *i.e.*, the fats of those groups receiving corn oil possessed the higher iodine numbers.

The experiments were not designed to investigate the effect of these supplements upon growth, but it is interesting to note that the animals receiving choline grew much better and were healthier in appearance than the other rats. Best *et al.* (19) have already noted a similar effect of choline; McHenry (20) has reported more extended studies of this and has discussed the beneficial influence of choline on growth.

Scaly tail and occasionally other signs usually taken as indicative of essential fatty acid deficiency (21, 22) were sometimes observed (particularly in Series A). Some of the rats receiving corn oil showed similar scaly tails. It is worthy of note that those groups receiving choline, even in the absence of corn oil, displayed little or no sign of the disease. The inclusion of "non-essential fat" apparently aggravated the condition, for the scaliness was most marked in Group 4, Series B. The real nature of the deficiency observed in these experiments is not understood. The possibility should not be overlooked that the various supplements used may have caused alterations in the intestinal flora, with consequent changes in the B vitamins and other metabolites synthesized in the gut (23).

The mortality of those rats not receiving choline was quite high due to the development of hemorrhagic kidneys. A few of the surviving rats which had received no choline had cirrhotic livers (confirmed by examination of sections) and all of them had "frosted" or badly pitted kidneys.

A few of the rats in the basal group of Series B, which died of hemorrhagic kidneys, had also areas of hemorrhage which followed roughly the distribution of the pancreas. This phenomenon was not noticed in any of the other groups.

Before any interpretation of the findings can be attempted, it will be necessary to determine whether the nullifying influence of corn oil upon the action of inositol is due to the essential fatty acids contained therein or whether some other component is involved. The problem is under investigation in this laboratory.

The authors wish to thank Professor C. H. Best for helpful criticism and advice in the preparation of the manuscript. Grateful acknowledgment is made to Miss M. O'Grady and Dr. C. S. McArthur for valuable help with the analytical work involved, and to Dr. T. F. Nicholson of the Department of Pathological Chemistry for examining a large number of tissue sections. Part of the expenses of this investigation has been defrayed by a grant from the Banting Research Foundation.

SUMMARY

1. Corn oil obliterates the lipotropic action of inositol under the dietary regimen used.

2. Choline brings about a greater reduction of cholesterol esters than does inositol under the conditions used.

3. Liver phospholipid is practically unaffected by the removal of choline, inositol, corn oil, or "non-essential fat" from the diet. The addition of these factors singly or in pairs did not change appreciably the amount of phospholipid in the liver.

BIBLIOGRAPHY

1. Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 107 (1936).
2. Welch, A. D., *J. Biol. Chem.*, **137**, 173 (1941).
3. Stetten, D., Jr., *J. Biol. Chem.*, **138**, 437 (1941).
4. Anderson, R. J., and Roberts, E. G., *J. Biol. Chem.*, **89**, 599 (1930).
5. Folch, J., and Woolley, D. W., *J. Biol. Chem.*, **142**, 963 (1942).
6. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, **24**, 128 (1944).
7. Engel, R. W., *J. Nutr.*, **24**, 175 (1942).
8. Birch, T. W., *J. Biol. Chem.*, **124**, 775 (1938).
9. Salmon, W. D., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **133**, p. lxxxiii (1940).
10. Beveridge, J. M. R., *Science*, **99**, 539 (1944).
11. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).
12. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 405 (1943).
13. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).
14. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.*, **150**, 315 (1943).
15. Sinclair, R. G., and Dolan, M., *J. Biol. Chem.*, **142**, 659 (1942).
16. King, E. J., *Biochem. J.*, **26**, 292 (1932).
17. Beeston, A. W., Channon, H. J., and Wilkinson, H., *Biochem. J.*, **29**, 2659 (1935).
18. Best, C. H., Channon, H. J., and Ridout, J. H., *J. Physiol.*, **81**, 409 (1934).
19. Best, C. H., Huntsman, M. E., McHenry, E. W., and Ridout, J. H., *J. Physiol.*, **84**, 38 P (1935).
20. McHenry, E. W., *J. Physiol.*, **85**, 343 (1935).
21. McAmis, A. J., Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, **82**, 247 (1929).
22. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **82**, 345 (1929).
23. Mitchell, H. K., and Isbell, E. R., *Univ. Texas Pub., No. 4237*, 125 (1942).

J. Nutrition 26:161-173, Aug. '43

THE EFFECTS OF PANTOTHENIC ACID AND INOSITOL ADDED TO WHOLE WHEAT BREAD ON EVACUATION TIME, DIGESTION AND ABSORPTION IN THE UPPER GASTRO-INTESTINAL TRACT OF DOGS[1]

C. G. BLY, F. W. HEGGENESS AND E. S. NASSET
Department of Vital Economics, University of Rochester, Rochester, New York

TWO FIGURES

(Received for publication January 18, 1943)

INTRODUCTION

In view of the recognized importance of the B-complex vitamins on gastrointestinal function, and on the basis of the favorable results on carbohydrate digestion and absorption with pantothenic acid indicated by Russell and Nasset ('41), it was felt that an expanded study should be made of the effect of this and other factors on the digestive tract. While McKibbin et al. ('40) and Schaefer et al. ('42) have mentioned alimentary canal changes in pantothenic acid deficiency, no report has included an extended study on the chronic effect of pantothenic acid deficiency on gastrointestinal function.

Our object was to study, in jejunostomized dogs, a chronic pantothenic acid deficiency with regard to gastrointestinal motility as well as carbohydrate and protein digestion and absorption.

EXPERIMENTAL

The procedures employed were similar to those outlined by Russell and Nasset ('41) and Lambooy and Nasset ('43). In adult mongrel dogs, Maydl jejunostomies were established

[1] Supported in part by a grant from the Continental Baking Company.

approximately 50 cm. caudad from the ligament of Treitz. At least 12 hours after last food (20 hours in the deficiency periods), the dogs were placed in a standing position in a stall and supported so as to provide the least possible discomfort. A two-lumened catheter with a rubber balloon on the end was introduced through the fistula well into the lumen of the gut. When the catheter was in place, the balloon was inflated through the small lumen with approximately 10 cc. of air, thus minimizing the loss of food past the catheter. Through the larger lumen of the catheter, the chyme, as it passed the enterostomy, was drawn by a negative pressure of 12 cm. of water into an appropriate receiving vessel.

After a pre-feeding collection period of at least ½ hour to make certain that the intestine was free of food, a weighed, analyzed test meal was fed. Collections of the chyme from the receiving vessel were made at approximately half-hour intervals, weighed, and the pH determined. The collections were then placed in a boiling water bath for 15 minutes to destroy all enzyme activity, pooled for each experiment, and stored at 5°C. until the analyses were made.

The following three methods were used to determine when the end of the experiment had been reached; in other words, to insure that the total emptying time required for complete passage of the test meal from the mouth to the catheter had elapsed: (1) the occurrence of a negative Benedict's qualitative sugar test on the collection; (2) the low weights of the half-hour collections; (3) the increase of pH occurring toward the last part of the experiment. The runs were continued for an additional hour or more to make certain that the intestine was empty. Since the emptying time and gastrointestinal motility must bear a fairly close, inverse relationship to each other, the former may be taken as a measure of the latter.

The basal diet which dog 1 received to establish a normal control consisted of water- and alcohol-extracted casein, 25%; alcohol-extracted sucrose, 41%; hydrogenated vegetable oil,[2] 18%; bone ash, 4%; dried brewers' yeast, 10%; and salts, 2%. The salts were those of the Phillips salt mixture as modified by Arnold and Elvehjem ('39), plus cobalt according to Frost et al. ('41).

[2] Primex.

The experimental diet consisted of a whole wheat bread made from flour prepared by the Earle flotation process. This bread was obtained fresh daily and fed moistened with water, about 400 gm. of bread being consumed. No difficulty was experienced in getting the dogs to eat the bread, and no dislike for it was ever evidenced, even after several months. The composition of the diet is given in table 1, the vitamin content having been determined by Sealock and Livermore ('43).

TABLE 1

Composition of experimental diet.

BREAD AS EATEN	VITAMIN CONTENT
	(μg./gm. fresh bread)
N 1.67% (protein 9.63%)	Thiamine — 2.73 (fermentation method)
	Riboflavin — 2.45 (rat growth)
Carbohydrate (by hydrolysis) 38.4%	Pyridoxine — 3.14 (rat growth)
	Pantothenic acid — 5.2 (bacterial growth)
	Inositol — 644.0 (yeast growth)
	Niacin — 30.0 (bacterial growth)

The dogs were fed the diet plus any vitamin supplements six times per week and always on the day preceding an experiment. Any dog suspected of coprophagy was muzzled in such a way as to prevent this, yet to permit the dog to drink water, which was provided ad libitum. The general program of feeding was to produce the deficiency, add the pantothenate supplement, reproduce the deficiency by removing the supplement, repeat the test of pantothenate supplement, and then testing the effects of other factors, including pyridoxine and inositol. The periods of deficiency and supplementation were alternated several times to permit detection of progressive changes.

In all periods the diets were supplemented with adequate amounts of the oil soluble vitamins A, D, E, and K, as follows: A, 200 I.U./kg./day; D, 10 I.U. kg./day; E, 1 mg./kg./day; K, 1 mg./kg./day.

The test meal consisted of 100 gm. of thoroughly dried and ground experimental diet, and was fed with 250 cc. of water at the beginning of each experiment, after the pre-feeding collection had been made. If a dog on any day refused to eat the entire test meal or if, for any reason, such as undue excitement or restlessness, the dog was considered not to be in a normal state, the experiment was discarded.

Analyses were made both for reducing sugar and total carbohydrate by the Shaffer-Somogyi ('33) method, and for diffusible and total nitrogen by the Kjeldahl method. From these data and from analysis of the test meal the amounts of carbohydrate and protein which had been digested and absorbed were calculated.

RESULTS

1. Motility

It was found that the gastrointestinal motility decreased in two dogs maintained on the whole wheat bread diet supplemented only with the oil soluble vitamins, and that additional pantothenic acid was necessary for the proper maintenance of that motility.

Dog 1 showed an average emptying time of 5.9 hours for the basal period of 4 weeks on the complete basal diet (fig. 1). When the animal was fed the experimental diet, the emptying time gradually increased in 7 weeks to an average of 11.8 hours. The administration of calcium pantothenate[3] at a level of 220 mg. per kilogram of body weight per day was begun after 9 weeks on the experimental diet. During the week following the beginning of administration of the vitamin, the emptying time decreased from an average of 11.8 hours (three experiments) to a normal average of 5.0 hours (two experiments). Using Fisher's ('38) t test the probability of this difference occurring by chance was calculated to be less than 1 in 100. Unfortunately, this animal later required a repair

[3] Kindly supplied by Merck and Company.

operation due to a closing fistula and died post-operatively. Death was not ascribed to the deficiency.

Dog 2, having been on the experimental diet nearly a month before experiments were begun, was already slightly deficient. The emptying time for the next 3 weeks increased to an

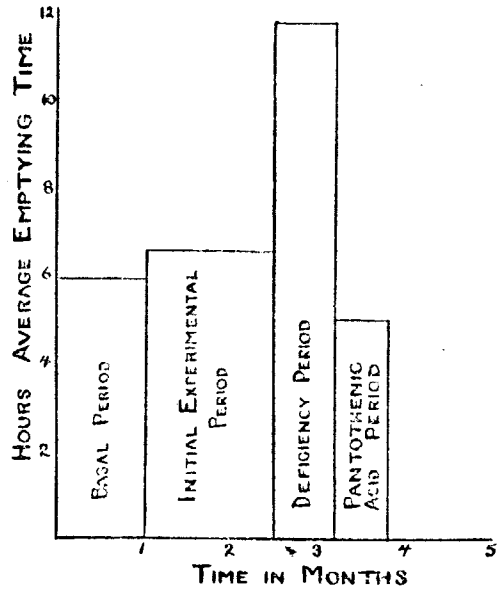


Fig. 1 Average emptying times for dog 1.

average of 10.4 hours (fig. 2). Within a week of calcium pantothenate administration at the same level as for dog 1, the emptying time dropped to a normal average of 5.6 hours over a period of a month. Upon removal of the pantothenate supplement the emptying time again gradually increased, reaching an average of 12.2 hours in 2½ months. Administration of the pantothenic acid at two later periods when the dog was in the deficient state resulted in each case in a prompt return of the motility to normal. On the basis of these combined periods the average emptying time during the deficiency periods was 11.5 hours (fourteen experiments) as compared to 6.1 hours for the vitamin control periods (nine experi-

ments). Again, the calculated probability of chance was less than 1 in 100. Hence, a deficiency of pantothenic acid in one period with dog 1 and in three different periods with dog 2 resulted in an average increase in emptying time above normal of 100% and 80%, respectively (figs. 1 and 2). Addition of 220 µg. of calcium pantothenate per kilogram per day restored the motility to normal. It appears likely, therefore, that the adult dog's daily pantothenic acid requirement lies somewhere

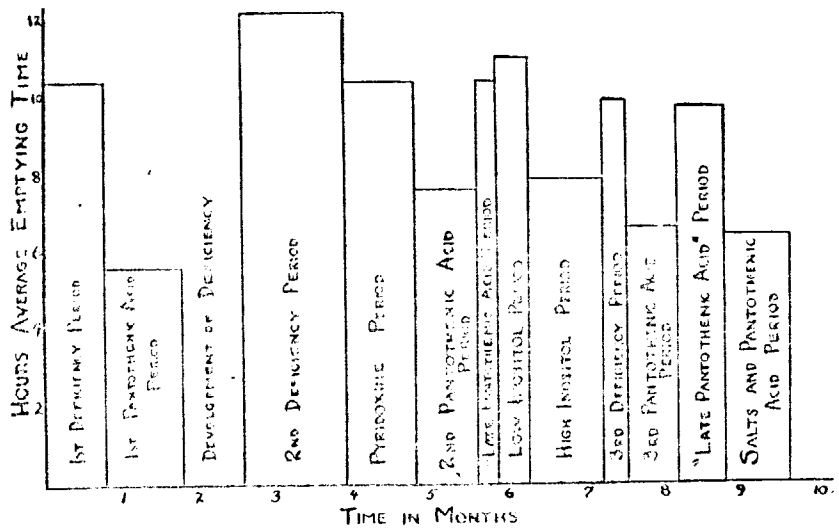


Fig. 2 Average emptying times for dog 2.

between 130 µg./kg. of body weight, the amount supplied by the bread, and 350 µg./kg. of body weight.

During pyridoxine administration (40 µg./kg./day) for a period of 1 month no significant effect on the deficient G. I. function could be demonstrated, the average emptying time for the period being 10.4 hours for four completed runs, or 60% over the basal level. In three other attempted runs, the dog vomited, having already retained the test meal in the stomach (very small collections, if any, through the catheter) for 7–10 hours after feeding. During this period healings of a lesion on one leg and of a lesion in the mouth were noted,

though these lesions had not been ascribed to a pyr[illegible] deficiency.

Martin et al. ('41) mentioned constipation in inositol-deficient mice, and reported that in dogs on a constipating though nutritionally adequate diet, inositol, as studied roentgenologically, had a marked stimulatory effect on intestinal peristalsis. Anderson ('16) had noted that inositol dosing caused a transitory diarrhea in man and dogs. Woolley ('42) has reported the intestinal synthesis of inositol in mice. He also reported that in vitro synthesis by intestinal flora did not proceed in the absence of pantothenic acid, and again ('41) that when sufficiently high levels of pantothenic acid were fed, alopecia frequently did not appear in inositol-deficient mice. Hence, it was thought that inositol might be tried to determine whether or not pantothenic acid acted indirectly by stimulating inositol production.

Subcutaneous administration of inositol (up to 33 mg./day) had no effect on the reduced intestinal motility (fig. 2), as would be expected with the large (though partially unavailable as phytin) content of inositol already present in the bread diet (table 1). Subsequent oral administration of up to 500 mg. per day of inositol resulted in reduction in the emptying time, one experiment being only 4½ hours long. However, as Anderson had noted with a normal diet, the effect was transitory and the length of the time again increased to over 10 hours in 2 weeks.

After 5 to 6 months on the bread diet, dog 2 began to show the effects of a secondary deficiency. After 3 weeks or so of the pantothenic acid supplementation, the emptying time tended to increase, despite continued administration of the vitamin. Increasing the dosage of the vitamin had no effect on this tendency of the "late pantothenic acid" runs. A complicating salt deficiency seemed probable; hence, 8 gm. per day of the basal salt mixture was added to the diet of dog 2 which was already receiving 3.30 mg. of pantothenic acid per day. Within a few days the emptying time decreased to an average of 6.2 hours (five experiments) — a value nearly

identical with the early value for pantothenic acid alone (fig. 2). It was concluded that there had been a complicating salt deficiency which had grown more and more severe over the months the animal was kept on the experimental diet.

Other than the effect on motility, there were interesting and possibly significant results. During the first deficiency period for each dog, as the emptying time increased, the normal sweet smell of chyme was replaced by a foul odor identified as that of hydrogen sulfide. This was not due to back seepage of gas from the colon of the atonic gut because, upon standing, the concentration of the gas in a given collection markedly increased. The odor of the H_2S disappeared during the periods of pantothenic acid feeding, but reappeared in dog 2 when the vitamin supplement was omitted from the diet. The muzzle worn by the dog eliminated the possibility of coprophagy. From twelve deficiency experiments, an average of 0.4 mg. of H_2S per experiment was obtained by the use of an aeration train. Pyridoxine was ineffective in reducing the amount of H_2S produced.

Other observations tended to fit in with a general gastrointestinal atony. During the deficient periods, the "buttons" of the fistulae became notably flabby and atonic, and leaks from the fistulae developed.

2. Digestion and absorption of carbohydrate and protein (see table 2)

It was found, in general, that the average total amounts of carbohydrate and protein digestion and absorption stayed remarkably constant during the different periods, the exceptions being the periods of the high inositol supplementation and of the salts and pantothenic acid supplementation. Marked differences in the rates were found, however, calculations being based for dog 1 on the eight basal runs, and for dog 2 on the nine runs of early pantothenic acid supplementation.

In both dogs, the average rates of carbohydrate and protein digestion and absorption show marked and statistically significant decreases during pantothenic acid deficiency. In dog 1

the digestion rates dropped to only one-half of the normal, while the absorption rates dropped to less than half. The probability of this difference being due purely to chance is less than 1 in 100. The data for dog 2 show similar differences, though not quite as great, for the rates of digestion and absorption between the average of the three different periods of pantothenic acid deficiency and that for the three corresponding periods of pantothenic acid supplementation (figs. 1 and 2, table 2). The differences in rate between basal and pantothenic acid periods in dog 1 are not significant. Pyridoxine supplementation did not alter the course of the deficiency.

Inositol exhibited a marked influence on the rates of carbohydrate and protein digestion and absorption. Relatively small doses (up to 33 mg./day) had no significant effects. Large oral doses (up to 500 mg./day or 32 mg./kg./day), however, exerted marked effects. The rate of carbohydrate digestion dropped to 58%, and that of protein digestion to 44% of the average rate in the pantothenic acid period; in each case this was lower than the rates of the deficiency periods. The average rates of absorption dropped much more, that of carbohydrate to 37% and that of protein to only 17% of the rate in the period on pantothenic acid supplement, both obviously highly significant decreases.

The irritating effect of inositol on the G. I. tract seems to be well-illustrated here, although an attempt was made to minimize the cathartic action by giving the inositol only with the regular daily meal and not with the test meal. A study of the protein absorption reveals this effect most clearly. The very low average total absorption and rate of absorption, coupled with the fact that two of the four analyzed runs showed rates of $-.3\%$/hour and -1.2%/hour absorption, respectively, indicate marked stimulation of secretion, and perhaps diminished capacity to absorb water, resulting in the recovery of more nitrogen than had been ingested. This seems confirmed by the significantly larger total collection volumes

(table 3). Wasteneys and co-workers ('41) have briefly discussed the difficulty of studying the true or absolute digestion and absorption of an ingested protein. It is of interest to note (table 3) that the ratio of total recovered nitrogen to the total collection volume remains relatively constant, even with inositol, showing a practically linear relationship throughout. Since digestive secretions contain little or no reducing substance, no complication from this cause enters into the interpretation of the data for carbohydrate.

TABLE 3

Average total collection volume and recovered nitrogen.

EXPERIMENT		TOTAL NITROGEN ABSORPTION		TOTAL COLLECTION VOLUME	NITROGEN RECOVERED IN COLLECTION
Type	Number				
		gm.	*% /hr.*	*cc.*	*mg./cc.*
Pantothenic acid supplement	9	1.08	6.7	450	3.6
Deficiency	14	1.00	3.4	500	3.3
Inositol	4	.24	1.1	705	3.2

Paradoxically enough, the dog lost no weight during the month of inositol supplementation and the accompanying poor absorption but rather showed a slight gain, the average weight during this period being 16.3 kg., and during the other periods, 15.7 kg. This suggests higher, compensatory absorption of the daily diet in the lower jejunum and ileum.

The whole response to inositol does not appear to be that of the relief of a deficiency of that substance, but rather, of a normal effect superimposed upon an unrelated deficiency.

In the "late pantothenic acid" runs discussed with regard to motility, the rates of digestion and absorption dropped back towards deficiency levels again, showing the existence of a long progressive secondary deficiency. Addition of salts did not relieve this deficiency, although the motility returned promptly to normal. Though the rates of carbohydrate digestion and absorption and protein digestion showed slight improvement, the total amounts of carbohydrate and protein digested and absorbed were lower, especially in the case of protein absorption.

TABLE 2

Results on digestion and absorption.

DOG NO. AND PERIOD	NO. OF RUNS	LENGTHS (HRS.) OF EMPTYING TIMES	CARBOHYDRATE						PROTEIN					
			Digestion			Absorption			Digestion			Absorption		
			Total (gms.)	% /hour	% from basal	Total (gms.)	% /hour	% from basal	Total (gms. N)	% /hour	% from basal	Total (gms. N)	% /hour	% from basal
Number 1														
Basal[1]	8	5.9	30.8	10.1	....	35.5	10.4	....	2.16	14.3	....	1.04	6.9	....
Deficiency	3	11.8	34.0	5.2	—48	33.4	4.4	—58	2.20	7.1	—50	.98	3.1	—55
Pantothenic acid supplement	2	5.0	32.7	13.0	+29	29.5	9.9	—5	2.28	17.6	+23	.94	7.2	+4
Number 2														
Pantothenic acid supplement[2]	9	6.4	36.4	9.8	....	34.7	8.4	....	1.96	12.3	....	1.08	6.7	....
Deficiency	14	11.5	37.9	6.2	—37	37.6	5.4	—36	1.90	6.5	—47	1.00	3.4	—49
Pyridoxine supplement	4	10.4	36.4	6.5	—34	38.2	6.3	—25	1.81	6.8	—45	.99	3.5	—48
Inositol (small amts.) suppl.	4	11.0	40.0	7.1	—28	35.7	6.3	—35	1.65	6.4	—48	.96	3.7	—47
Inositol (large amts.) suppl.	4	7.9	26.4	5.7	—42	16.8	3.1	—63	1.06	5.4	—56	.24	1.1	—83
Number 2														
"Late pantothenic acid" runs	6	9.7	25.2	6.2	—37	31.4	5.1	—39	1.61	6.4	—48	.96	3.4	—49
Salt supplement	5	6.1	31.9	8.8	—10	23.2	5.9	—30	1.97	8.1	—34	.98	3.4	—51

[1] Exclusive of this period, experimental diet was used in the daily feeding. Experimental test meals were used in all the runs. [2] 500 mg./kg./day.

CONCLUSIONS

In enterectomized dogs maintained exclusively on a peeled whole wheat bread diet, a severe deficiency was produced in the course of 2 to 3 months. This was characterized by an approximately 50% decrease in gastrointestinal motility, accompanied by 40–60% decreases in the rates of carbohydrate and protein digestion and absorption. The almost immediate effect of adding a supplement of 220 µg. of calcium pantothenate per kilogram of dog weight per day to this diet was the return of these functions to normal in every case. The total digestion and absorption remained practically the same in the normal deficient states.

Pyridoxine was ineffective in altering the course of the deficiency.

Inositol acted more as a cathartic, its effect being apparently superimposed on the deficiency. While the motility was temporarily improved, the total amounts and rates of digestion and absorption were greatly decreased. A suggestion of synergism between inositol and pantothenic acid was not confirmed by this method.

After 5 to 6 months on the experimental diet, a secondary deficiency was noticed, characterized by diminished effectiveness of continued pantothenic acid supplementation to maintain the normal functions of motility, digestion and absorption. Adding a salt supplement, together with the continued pantothenic acid supplement, resulted in a prompt return of the motility to normal but only in very slight improvements in digestion and absorption rates.

LITERATURE CITED

ANDERSON, R. J. 1916 The utilization of inosite in the dog. J. Biol. Chem., vol. 25, p. 391.

ARNOLD, A., AND C. A. ELVEHJEM 1939 Influence of the composition of the diet on the thiamin requirement of dogs. Am. J. Physiol., vol. 126, p. 289.

FISHER, R. A. 1936 Statistical methods for research workers. Oliver and Boyd, London.

FROST, D. V., C. A. ELVEHJEM AND E. B. HART 1941 A study of the need for calcium pantothenate in dogs on heated diets. J. Nutrition, vol. 21, p. 93.

LAMBOOY, J. P., AND E. S. NASSET 1943 The inadequacy of eight synthetic B vitamins for the nutrition of puppies — unknown factor (factors) in yeast and probably liver. J. Nutrition, vol. 26 (in press).

MCKIBBIN, J. M., S. BLACK AND C. A. ELVEHJEM 1940 The essential nature of pantothenic acid and another alkali labile factor in the nutrition of the dog. Am. J. Physiol., vol. 130, p. 365.

MARTIN, G. J., M. R. THOMPSON AND J. DE CARVAJAL-FORERO 1941 Influence of inositol and other B-complex factors upon the motility of the G. I. tract. Am. J. Dig. Dis., vol. 8, p. 291.

RUSSELL, R. S., AND E. S. NASSET 1941 The effects of various vitamin supplements and of whole yeast on the digestion and absorption of the carbohydrate of a complete diet. J. Nutrition, vol. 22, p. 287.

SCHAEFER, A. E., J. M. MCKIBBIN AND C. A. ELVEHJEM 1942 Pantothenic acid deficiency studies in dogs. J. Biol. Chem., vol. 143, p. 321.

SEALOCK, R. R., AND A. LIVERMORE 1943 The vitamin B-complex of peeled wheat bread. J. Nutrition, vol. 25, p. 265.

SHAFFER, P. A., AND M. SOMOGYI 1933 Copper-iodometric reagents for sugar determinations. J. Biol. Chem., vol. 100, p. 695.

WASTENEYS, H., B. F. CHOW AND R. HAMILTON 1941 Studies of digestion in the dog. Am. J. Physiol., vol. 135, p. 6.

WOOLLEY, D. W. 1941 Identification of the mouse anti-alopecia factor. J. Biol. Chem., vol. 139, p. 29.

——— 1942 Bacterial synthesis of inositol and its importance in the nutritional economy in mice. J. Bact., vol. 43, p. 5.

J. Nutr.
26: 477-485, 1943

LOSSES OF B VITAMINS DUE TO COOKING OF FOODS [1]

VERNON H. CHELDELIN,[2] ALETHEA M. WOODS AND ROGER J. WILLIAMS
University of Texas, Austin

(Received for publication March 22, 1943)

Much information has been obtained recently regarding the distribution of the various B vitamins in natural foodstuffs (Waisman and Elvehjem, '41; Lane, Johnson and Williams, '42; Cheldelin and Williams, '42). However, information is less complete regarding the amounts of these vitamins present in foods as they are actually eaten.

In a previous paper (Lane, Johnson and Williams, '42) the losses in thiamine due to cooking were determined for a large number of foods comprising the average American diet. The present investigation deals with similar losses in six other vitamins: riboflavin, nicotinic acid, pantothenic acid, biotin, inositol and folic acid.

EXPERIMENTAL

Selection and sampling of foods

Vegetables, fruits, milk and eggs were purchased in nearby markets in as fresh condition as possible. Meats were, with few exceptions, obtained from a single wholesale butcher so that it was possible in most cases to be assured of freshness of the samples. Fish were bought as frozen commercial products.[3] Perishable foods were kept at 0–5° C. until they were

[1] Presented before the Division of Biological Chemistry at the 104th meeting of the American Chemical Society, Buffalo, New York.

[2] Present address: Department of Chemistry, Oregon State College, Corvallis, Oregon.

[3] Birds-Eye.

prepared for assay. These preparations were made within a few hours after procurement of the samples.

Procedure

The microbiological assay methods developed in the University of Texas Laboratories (Williams, '41) have been employed throughout for each of the six vitamins herein discussed.

All food extracts were prepared by digestion with takadiastase and papain according to the general method described previously (Cheldelin, Eppright, Snell and Guirard, '42). This procedure has proved to be quite satisfactory for preparation of extracts of natural materials, although it has not been confirmed generally for use with cooked foods. It is possible that in certain cases vitamins which have become "bound" by cooking and thus rendered unavailable to microorganisms (or to the reagents used in various chemical tests) may still be available for animal nutrition. This is apparently true for thiamine in some tissues (Lane, Johnson and Williams, '42). Satisfactory animal assays are not available for several vitamins, however, and in the absence of further direct evidence we have chosen to employ the digestion procedure mentioned above. Vitamin losses which appear to be very large may warrant reexamination at a later time.

RESULTS

Tables 1 and 2 contain the details of cooking and the results of the analyses of samples of thirty foods before and after cooking for the six vitamins mentioned above.

Lane, Johnson and Williams ('42) have pointed out the difficulties in estimating the amounts of cooking waters to be used or discarded in the preparation of foods. In an effort to standardize these factors we have included cooking waters and meat juices as part of the cooked samples except in a few cases where the cooking waters were assayed separately and discarded. The values in table 2 therefore tend to represent maximum amounts of the various vitamins present after cooking.

TABLE 1

Details of the cooking of various foods.

FOOD	MANNER OF COOKING	TIME OF COOKING	WATER OR FAT ADDED	WEIGHT BEFORE COOKING	EDIBLE AFTER COOKING
Meats		*Minutes*	*gm.*	*gm.*	*gm.*
1.[1] Beef round	Fried in open pan	15	1	40	30
2. Beef liver	Fried in open pan	10–15	1	40	35
3. Beef liver	Fried, covered pan	10	16	94	75
4. Beef heart	Steamed	30	93	190	210
5. Beef heart	Steamed	30	50	100	150
6. Pork loin	Fried in open pan	10–15	0	40	29
7. Pork loin	Fried in open pan	10–15	0	45	28
8. Pork loin	Fried in open pan	10–15	0	68	43
9. Bacon	Fried in open pan	5	0	41	17
10. Bacon	Fried in open pan	3–5	0	22	13
11. Ham	Fried in open pan	15	0	54	30
12. Ham	Fried in open pan	15	0	59	37
13. Veal chop	Fried in open pan	10–15	0	42	30
14. Veal chop	Steamed	30	84	170	180
15. Lamb leg	Roasted, 300° F.	120	0	690	550
16. Mutton shoulder	Steamed	30	66	133	199
17. Chicken leg	Fried in open pan	10–15	0	41	35
18. Chicken leg	Fried in open pan	10–15	5	69	46
19. Chicken breast	Fried in open pan	10–15	0	31	29
20. Chicken breast	Fried in open pan	10–15	0	31	29
21. Salmon	Fried in open pan	20	3	111	97
22. Halibut	Fried in open pan	7–10	2	126	104
23. Halibut	Steamed	30	0	5	5
Vegetables					
24. Beets	Boiled, covered kettle	40	266	133	124
25. Cooking water					140
26. Beets	Steamed	40	190	380	570
27. Beet tops	Steamed	10	20	40	60
28. Cabbage	Steamed	30	25	100	125
29. Carrots	Steamed	30	25	50	75
30. Cauliflower	Steamed	20	120	120	240
31. Cauliflower	Steamed	30	50	100	150
32. Onions	Fried in open pan	20	29	208	53
33. Potatoes	Boiled, covered kettle	30	200	190	200
34. Cooking water					190
35. Potatoes	Boiled, covered kettle	20	360	180	210
36. Cooking water					210
37. Lima beans	Steamed	60	10	5	15
38. Okra	Steamed	20	15	30	45
39. Rice	Steamed	25	60	30	90
40. Sauerkraut	Steamed	30	0	5	5
41. Spinach	Steamed	10	50	100	150
42. Split peas	Steamed	40	100	50	150
43. Sweet potatoes	Baked	45–60	0	2200	1700
44. Turnips	Boiled, covered kettle	40	114	114	118
45. Cooking water					90
46. Turnips	Steamed	20	22	22	44
Fruit					
47. Apples	Boiled, open kettle	20–30	1300	3400	2300
Eggs and milk					
48. Eggs	Scrambled, open pan	10–15	8	150	139
49. Eggs	Scrambled, open pan	10–15	1	50	46
50. Eggs	Baked	30	0	100	100
51. Eggs	Scrambled, covered pan	5	0	100	94
52. Milk	Heated, glass dish	30	0	100	100
53. Milk	Heated, open metal pan	15	0	100	100

[1] The numbers in this column correspond to those in the first column of table 2.

TABLE 2 — *Losses of certain B vitamins during cooking* [1]

FOODS	RIBOFLAVIN			NICOTINIC ACID			PANTOTHENIC A
	Total content		Loss	Total content		Loss	Total content
	Before	After		Before	After		Before	After
Meats	μg.	μg.	%	μg.	μg.	%	μg.	μg.
1.[2] Beef round	88	69	22	1800	1700	5.5	190	130
2. Beef liver	1100	1300	0	4700	5200	0	2200	1900
3. Beef liver	2400	2300	4.2	14000	14000	0	8600	7400
4. Beef heart	1700	1700	0	15000	13000	13	3400	1900
5. Beef heart	890	950	0	8700	7400	15	2000	1400
6. Pork loin	130	87	33	1500	410	72	280	200
7. Pork loin	90	75	17	2300	1500	35	260	190
8. Pork loin	100	72	28	6200	4200	32	320	200
9. Bacon	43	51	0				110	110
10. Bacon	69	63	8.7	920	940	0	220	170
11. Ham	140	110	21	1800	1700	5.5	180	220
12. Ham	150	125	17	1900	2200	0	390	390
13. Veal chop	59	59	0	3000	2600	13	110	94
14. Veal chop	380	400	0	1200	1100	8.5		
15. Lamb leg	1700	1700	0	52000	42000	19	4100	3600
16. Mutton shoulder	330	330	0	5300	5300	0	570	570
17. Chicken leg	110	110	0	1800	1600	11	320	250
18. Chicken leg	170	160	5.9	2200	1800	18	360	240
19. Chicken breast	40	40	0	2900	2800	3.4	180	140
20. Chicken breast				3300	3400		170	160
21. Salmon	160	160	0	7100	6600	7.0	730	740
22. Halibut	110	86	22	9400	9200	2.1	140	140
23. Halibut	2.2	2.2	0	710	670	5.6	9.5	9.5
Vegetables								
24. Beets	51	42	18	740	340	54	170	130
25. Cooking water		10	0 [3]		270	18 [3]		9
26. Beets	230	230	0	2300	890	61	420	260
27. Beet tops	86	74	14	240	230	4.2	56	36
28. Cabbage	57	48	16	210	170	19	180	130
29. Carrots								
30. Cauliflower	150	130	13	780	610	22	970	970
31. Cauliflower	120	100	17	530	550	0	1000	940
32. Onions	50	37	26					
33. Potatoes	95	86	9.4	1600	1100	31		
34. Cooking water		14	0 [3]		490	0.6 [3]		
35. Potatoes	92	52	43	2600	1900	27		
36. Cooking water		40	0 [3]		700	0 [3]		
37. Lima beans	6.6	6.8	0	49	57	0	42	31
38. Okra	32	39	0	210	210	0	69	81
39. Rice	16	18	0	170	220	0	110	110
40. Sauerkraut	2.0	1.9	5.0	8.5	8.0	5.9	2.7	2.7
41. Spinach	220	160	27	510	430	16	160	150
42. Split peas	88	93	0					
43. Sweet potatoes	910	790	13	9980	9500	4.8		
44. Turnips	48	43	10	1100	660	40	160	94
45. Cooking water		8	0 [3]		440	0 [3]		54
46. Turnips				15	16	0		
Fruit								
47. Apples	580	430	25					
Eggs and milk								
48. Eggs	655	341	48					
49. Eggs	205	160	22					
50. Eggs	360	380	0	67	50	25	1400	1500
51. Eggs	360	380	0					
52. Milk	166	87	48					
53. Milk	166	130	22					

[1] The amounts of respective vitamins given in this table are those found in the number of g food analysed shown in the last two columns of table 1.

Losses of certain B vitamins during cooking[1] TABLE 2

BIOTIN			INOSITOL			FOLIC ACID		
Total content		Loss	Total content		Loss	Total content		Loss
Before	After		Before	After		Before	After	
μg.	μg.	%	mg.	mg.	%	μg.	μg.	%
0.88	0.33	63	18	16	11	42	8.7	79
15	18	60	22	15	32	130	110	15
						540	630	0
25	11	56	420	420	0	11	3.6	67
4.9	1.5	69	290	330	0	160	12	92
2.2	1.9	14	18	13	28	26	8.0	69
1.7	0.62	64	20	13	35	61	3.2	95
1.4	0.47	66	25	20	20	57	5.4	91
3.0	2.0	33	18	7.5	58	25	1.9	92
1.7	1.4	18	14	12	14	35	2.6	91
3.2	1.8	44	17	18	0	31	3.9	87
2.4	1.8	25	34	27	21	70	4.2	94
0.59	0.30	34	15	16	0	69	29	58
3.4	2.0	41	54	32	39	160	55	66
15	15	0	400	310	24	800	100	88
3.6	2.4	33	68	77	0	100	31	69
4.6	1.3	72				61	22	64
5.7	3.7	35	26	22	15			
2.0	1.4	30	10	10	0			
1.5	0.84	43	21	14	33	63	21	67
5.9	4.5	24	19	19	0	98	37	62
12	8.4	30	24	18	25	67	36	46
0.33	0.32	3	.7	.7	0	6.0	1.3	74
						120	7	94
							5	90[2]
1.1	1.1	0	80	43	46	170	50	71
1.1	1.0	9.1	8.3	3.9	54	84	22	74
2.4	2.8	0	95	62	35	65	5	92
			24	24	0	48	1.2	97
23	20	13	96	93	3.1	190	23	88
14	10	28	110	60	46	110	34	69
						190	14	93
							13	86[2]
						150	6	96
							4	93[2]
0.49	0.37	24	8.4	3.3	61	17	1.6	91
1.7	1.8	0	16	16	0	16	4.5	72
0.39	0.48	0	7.9	3.1	61	26	2.1	92
0.095	0.11	0	1.5	1.2	20	0.93	0.93	0
5.9	5.1	14	27	20	26	170	27	84
9.2	7.3	21	160	7.8	95			
						130	6	95
							8	89[2]
0.46	0.51	0	10	11	0	11	1.5	86
			19	20	0	110	38	65
4.3	4.5	0				110	76	31

[1] Numbers in this column correspond to those in the first column of table 1.
[2] Rinsing waters assayed separately and included with food samples.

Riboflavin

As has been pointed out previously (Williams and Cheldelin, '42), riboflavin losses during cooking tend to be greatest in the presence of light. Large losses, up to 48%, were incurred in the cooking of eggs, milk and pork chops.

Riboflavin losses sustained in the absence of light are, on the other hand, remarkably small. Steaming of meats or vegetables results, for the most part, in small or negligible losses of this vitamin. Similar observations have been made for meats by McIntire and coworkers ('43). The thermostability of riboflavin is indicated by the very slight destruction in baking sweet potatoes and roasting lamb. The contrast between losses in the presence and absence of light is especially marked for halibut, eggs and milk.

The opacity of many foods tends to prevent excessive destruction of riboflavin even in the light. Bacon, for example, fried for a relatively short time, retains essentially all of its riboflavin.

The apparent gains in riboflavin found with some samples are probably not significant. Substantial gains (greater than 10%) are often recorded with materials of relatively low potency where the accuracy of the assay method is poorest. In any case the assay method is probably accurate only to $\pm$ 10%. This qualification applies not only to riboflavin assays but to the others as well.

Nicotinic acid

Nicotinic acid is known to be quite stable to heat. Losses in cooking would therefore be expected to be slight. This is true for most of the samples listed in table 2, with the exception of pork chops, eggs and beets.

The small losses observed for meats are markedly lower than those reported by Waisman and Elvehjem ('41) and Dann and Handler ('42), using chemical methods of assay, although McIntire, et al. ('43) observed only slight losses in cooking of pork when microbiological assays were used. Since the results were obtained for different samples the reason for the discrepancies is not apparent. It is possible that chemical changes in the antipellagric factor which accompany heating may not be reflected in the microbiological assay.

Pantothenic acid

Pantothenic acid losses are seen from table 2, to be moderate to slight among vegetables and eggs, as well as among such meats as bacon, ham, mutton and fish which are here subjected to relatively mild heat treatment. Beef heart and beets are exceptions. Frying of beef or pork for longer periods of time results in loss of about one third of the vitamin present. The results with meats are in general agreement with the data of Waisman and Elvehjem ('41), although the present losses are somewhat smaller for pork.

Biotin

Biotin losses among vegetables are moderate, ranging from 28% in steamed cauliflower to no loss in beets, cabbage, okra, rice, sauerkraut, and turnips. There is likewise no loss of biotin in frying eggs.

Losses in biotin among meats are generally higher, reaching 72% for fried chicken leg. Severity of heat treatment does not seem to be directly responsible for increased losses, since beef heart loses about 60% by steaming 30 minutes, whereas roasting of lamb at 150° C. for 2 hours results in practically no loss of biotin.

Inositol

Inositol losses among meats are quite variable, but are moderate in most cases. In general smaller losses are observed among samples which have been subjected to relatively mild treatment such as steaming (mutton, heart, and halibut). The greatest loss (58%) is found for one sample of fried bacon.

In contrast to most of the other B vitamins, inositol losses in cooking of vegetables are often much greater than those encountered for meats. Losses are especially high among the

legumes, with split peas losing 95% of their inositol content. In view of the stability of inositol toward heat, this is surprising especially since the severity of heat treatment of the vegetables tested was much less than that for most of the meats. It seems likely, therefore, that the losses observed may be due to "binding" of the vitamins in various tissue combinations rather than to thermal destruction.

Folic acid

Of all the vitamins studied, folic acid showed the greatest loss due to cooking. With the exception of liver and sauerkraut, which retain the bulk of their folic acid content, losses among meats range from 46% in halibut to 95% in pork chops; losses in vegetables range from 69% in cauliflower to 97% in carrots.

The reasons for such large losses are not known. Although the vitamin is unstable to light, it is not readily destroyed by autoclaving in the dark in the pH range common to most foods. It seems likely that folic acid may become "bound" in tissues during cooking. This may be investigated further at a later time.

SUMMARY

Cooking losses as determined with microorganisms, have been determined in thirty foods for riboflavin, nicotinic acid, pantothenic acid, biotin, inositol and folic acid. These are summarized below.

VITAMIN	DEGREE OF LOSS DURING COOKING
Riboflavin —	Destroyed in variable amounts in presence of light; negligible losses in the dark.
Nicotinic acid —	Generally slight.
Pantothenic acid —	Moderate to slight in vegetables; somewhat larger (up to one third) in meats.
Biotin —	Moderate to negligible in vegetables; quite high in meats (up to 72%).
Inositol —	Generally moderate among meats, with steamed samples only slight; often very great (up to 95%) in vegetables, especially in legumes.
Folic acid —	Very great for most foods.

ACKNOWLEDGMENT

The initial portions of this work were carried out in the laboratory of Dr. R. R. Williams. We wish to acknowledge with thanks his cooperation and aid during various phases of the work. We are also indebted to Misses Margaret J. Hofer and Jean Taylor and to Messrs. Robert L. Lane and Gerald A. Johnson for conducting many of the assays, as well as to Research Corporation for their financial aid.

LITERATURE CITED

CHELDELIN, V. H., M. A. EPPRIGHT, E. E. SNELL AND B. M. GUIRARD 1942 Enzymatic liberation of B vitamins from animal and plant tissues. The University of Texas Publication, no. 4237, p. 15.

CHELDELIN, V. H., AND R. J. WILLIAMS 1942 The B vitamin content of foods. The University of Texas Publication, no. 4237, p. 105.

DANN, W. J., AND P. HANDLER 1942 The nicotinic acid content of meat. J. Nutrition, vol. 24, p. 153.

LANE, R. L., E. JOHNSON AND R. R. WILLIAMS 1942 Studies of the average American diet. I. Thiamine content. J. Nutrition, vol. 23, p. 613.

MCINTIRE, J. M., B. S. SCHWEIGERT, L. M. HENDERSON AND C. A. ELVEHJEM 1943 Retention of vitamins in meat during cooking. J. Nutrition, vol. 25, p. 143.

WAISMAN, H. A., AND C. A. ELVEHJEM 1941 The Vitamin Content of Meat. Burgess, Minneapolis.

WILLIAMS, R. J. 1941 The University of Texas Publication, no. 4137, p. 7.

WILLIAMS, R. R., AND V. H. CHELDELIN 1942 Destruction of riboflavin by light. Science, vol. 96, p. 22.

Univ. Micro;Dissertation Abstr. 19, 1541-2; 1959

STUDIES ON THE METABOLISM OF MYO-INOSITOL IN ANIMALS

(L. C. Card No. Mic 58-7470)

Robert Herman Coots, Ph.D.
The University of Wisconsin, 1958

Supervisor: Associate Professor Laurens Anderson

Two aspects of myo-inositol metabolism have been studied: (1) its reported role as an ascorbic acid-sparing agent and (2) the catabolism of myo-inositol-2-C^{14} in the rat.

The possibility that myo-inositol could substitute for or spare ascorbic acid in the diet of guinea pigs has been examined in three ways. First, myo-inositol was given to adult guinea pigs subsisting on a vitamin C-deficient purified diet--to some from the beginning of the experiment, to others after scurvy had developed. Second, essentially the same plan was followed with a scorbutogenic natural diet. Finally, young guinea pigs on a purified diet were allowed to become scorbutic, then supplied with levels of ascorbic acid that ranged from suboptimal to sufficient. Some of the animals on each of these levels of ascorbic acid were also given inositol. myo-Inositol did not have any detectable antiscorbutic activity in any of these experiments. The results have been published in J. Nutrition, 64: 167, 1958.

For the study of the in vivo catabolism of myo-inositol-2-C^{14} the compound was administered by intraperitoneal injection to normal adult male white rats. It was found that myo-inositol is incorporated intact into complex tissue components, such as phospholipids. It was also clearly shown that myo-inositol is vigorously metabolized to CO_2 by the rat, 20-40% of the administered dose being excreted into the respiratory CO_2 within 8 hours. Peak specific activity in the CO_2 is reached in 1 to 2 hours.

Most of the effort in this phase of the work was devoted to the study of the inositol to glucose conversion. Incorporation of label into the glycogen was found to be quite slow, periods greater than 3 hours being required to attain appreciable specific activity. The specific activity of each carbon of the glucose from the glycogen was determined using a Leuconostoc mesenteroides fermentation with subsequent chemical degradation of the fermentation products. The label distribution was found to be predominantly 1,6, with the label divided almost equally between these positions.

Radioactive glucuronic acid was isolated from the urine by column chromatography and identified by paper chromatography. The specific activity and distribution of label in the urinary free glucuronic acid indicated that, although some of this substance is formed from glucose, most of it arises more directly from inositol. The urinary glucuronic acid is apparently entirely of the D-form, and not a mixture of isomers as indicated by the in vitro studies of Charalampous (J. Biol. Chem., 228: 1, 1957).

The data which were obtained were considered in the light of a scheme for glucuronic acid metabolism presented by Burns (J. Am. Chem. Soc., 79: 3604, 1957). Glucose produced by these reactions should be predominantly labeled in positions 1 and 6; however, it is felt that carbon 6 would have significantly more label than carbon 1. Possible reasons for the deviation of the observed label distribution from theoretical are discussed.

The evidence cited suggests that conversion to glucose by way of glucuronic acid plays a role in the over-all metabolism of myo-inositol. However, the fact that respiratory CO_2 reaches a peak in specific activity much sooner than glycogen, and the discrepancy between the observed and the expected label distribution in the glucose strongly suggest the existence of one or more other pathways for this metabolism. A preliminary account of the results has been published in Biochim. et Biophys. Acta, 28: 666, 1958. Microfilm $2.00; Xerox $5.00. 100 pages.

A NOTE ON THE INFLUENCE OF INOSITE UPON THE EXCRETION OF PHENOL IN THE DOG.

By HARRY DUBIN.

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia.)

(Received for publication, November 27, 1916.)

As a result of his work on the utilization of inosite, Anderson[1] found that in the dog, after feeding a known amount of inosite, he could recover in the feces up to 77 per cent of the amount, while the urine contained only minimal amounts. In man[2] 9 per cent was recovered in the urine while none at all could be demonstrated in the feces. The question as to what happens to the rest of the inosite is left open.

In accordance with the belief that inosite is a hexahydroxybenzene[3] and that inosite undergoes bacterial fermentation in the intestine it seems plausible to consider the possibility that the benzene fraction may be split off, thus giving rise to phenol.[4] Consequently it was thought worth while to try to demonstrate an increase in the phenol content of the urine after feeding inosite.[5]

The results of such feeding are shown in the table, and it is evident that no "extra" phenol appears in the urine upon the ingestion of inosite, so that the rôle which this substance plays in metabolism is still in doubt.

[1] Anderson, R. J., *J. Biol. Chem.*, 1916, xxv, 391.

[2] Anderson, R. J., and Bosworth, A. W., *J. Biol. Chem.*, 1916, xxv, 399.

[3] Hammarsten, O., and Hedin, S. G., Physiological Chemistry, New York, 7th edition, 1915, 579.

[4] Folin O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.

[5] The inosite was obtained through the kindness of Dr. Anderson.

Influence of Inosite on the Excretion of Phenol in the Dog.

Date	Total N.	Phenol.				Inosite.			
		Free.	Total.	Free.	Conjugated.	Fed.	Recovered.		
							Urine.	Feces.	Percentage of amount fed.
1916		gm.	gm.	per cent	per cent	gm.	gm.	gm.	
Oct. 15	14.82	0.203	0.267	76.2	23.8				
" 16	14.89	0.200	0.270	74.2	25.8				
" 17	14.99	0.208	0.277	75.2	24.8	5.0	None.		
" 18	14.75	0.203	0.272	74.8	25.2			1.875*	37.5
" 19	14.84	0.202	0.267	75.8	24.2	20.0	0.111		
" 20	15.00	0.205	0.276	75.3	24.7			8.234†	41.2

* Feces of 17th and 18th.

† Feces of 19th and 20th.

As for the actual feeding and recovery of inosite, the results obtained corroborate those of Anderson.[1]

Proc. Soc. Exp. Biol. Med. 63, 479-80, 1946

15643

Effects of Massive Doses of P-Aminobenzoic Acid and Inositol on Reproduction in the Rat.

BENJAMIN H. ERSHOFF.

From the Emory W. Thurston Laboratories, Los Angeles, Calif.

A few years ago Martin[1] found that although rats grew adequately on purified rations containing 6 B complex factors (thiamine, riboflavin, pyridoxine, choline, nicotinic acid and calcium pantothenate), addition of inositol resulted in retardation of growth and a number of early deaths. Similar adverse effects were noted following the administration of *p*-aminobenzoic acid but not when *p*-aminobenzoic acid and inositol were fed concurrently. Subsequent studies with rats have not confirmed these results,[2] although Sure[3] observed a toxic effect of inositol on lactation and Ershoff and McWilliams[4] noted reduced fertility when inositol was fed along with purified rations containing both *p*-aminobenzoic acid and sulfaguanidine. In the present experiment rats were raised to maturity on purified rations containing massive doses of *p*-aminobenzoic acid or inositol and the adequacy of such diets for reproduction was determined.

Procedure and Results. Forty-two female rats of the Long-Evans strain were selected at 23 days of age and an average weight of 41.8 g. They were kept in metal cages with raised screen bottoms to prevent access to feces and were fed *ad libitum* the 7 diets listed in Table I (6 animals per group). They were weighed weekly, and vaginal smears were taken daily from the 48th day. After the 60th day the animals were bred to males of proven fertility.

Growth. No significant difference in rate of growth or gross appearance was observed except perhaps in the rats fed diet C. After 60 days the animals on this diet averaged 173.8 g in weight; average weights for those on the other diets ranged from 184.2 g on diet G to 204.8 g on diet F.

Reproduction. No significant difference in reproductive performance was observed.

TABLE I.
Composition of Experimental Diets.

Dietary component	A	B	C	D	E	F	G
Casein*	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Salt mixture†	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Sucrose	65.5	64.5	63.5	64.5	63.5	65.5	55.5
p-aminobenzoic acid		1.0	2.0		1.0		
Inositol				1.0	1.0		
Folic acid						0.0005	
Yeast‡							10.0

To each kg of diets A-F, were added 80 mg thiamine hydrochloride, 20 mg riboflavin, 20 mg pyridoxine hydrochloride, 100 mg calcium pantothenate, 100 mg nicotinic acid, 5 mg 2-methyl-naphthaquinone and 1.2 g choline chloride. To each kg of diet G were added 5 mg 2-methyl-naphthaquinone and 1.2 g choline chloride. In addition each rat on diets A-G received the following supplement 3 times weekly: 500 mg cottonseed oil (Wesson), 1.0 mg alpha-tocopherol, and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 50 U.S.P. units of vitamin D.‖

* Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

† Sure's Salt Mixture No. 1.5

‡ Brewers' type yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

‖ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

[1] Martin, Gustav J., *Am. J. Physiol.*, 1942, **136**, 124.

[2] Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 190.

[3] Sure, Barnett, *J. Nutrition*, 1943, **26**, 275.

[4] Ershoff, B. H., and McWilliams, H. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 227.

[5] Sure, Barnett, *J. Nutrition*, 1941, **22**, 499.

All the rats cast litters, although in 3 instances the initial breeding proved unsuccessful. Number of young per litter varied from 2 to 13, with averages between 7.2 and 8.4 on the several diets. An average of 0.8 to 1.2 young per litter were born dead (omitting one litter on diet C which consisted of 9, all born dead). In all groups the young averaged 5 to 5½ g at birth and appeared grossly to be normal in all respects.

Lactation. At birth litters were reduced to 6 each. The young were nursed and weaned on all diets except E and F. On each of diets B, D and G, 3 mothers successfully nursed their young, which were weaned at 21 days of age at average weights of 36 to 41 g. Two litters on diet A were nursed, but only 5 of the 12 young survived until weaning (average weight at weaning, 22.8 g). The one litter weaned on diet C consisted of 3 survivors, average weight 24.6 g.

The findings indicate that the massive doses of inositol or *p*-aminobenzoic acid exerted no deleterious effects on growth or reproduction and that lactation may occur on diets containing 1% *p*-aminobenzoic acid or inositol. The cause of the poor lactation is unknown, but deficiency of folic acid was probably not a factor. To judge from the work of Maynard and Rasmussen,[6] failure of lactation may have been due in part to insufficiency of dietary fat.

Summary. No adverse effects on growth or reproduction were noted in female rats fed purified rations containing 1% *p*-aminobenzoic acid or inositol. Lactation was inadequate both in control and experimental rations, although some young were weaned on each of the above diets.

[6] Maynard, L. A., and Rasmussen, E., *J. Nutrition*, 1942, **23**, 385.

Proc. Soc. Biol. Med. 54:89, 1943

14315

Lipotropic Action of Inositol.

J. C. Forbes.

From the Medical College of Virginia, Richmond, Virginia.

It has been shown by Gavin and McHenry[1] that the fatty livers which result from the administration of biotin to rats on a high carbohydrate-low protein diet are very resistant to the lipotropic action of choline but respond readily when inositol is given in addition. A recent publication by McHenry and his associates[2] shows that inositol is apparently inactive against the fatty livers which develop when thiamine is the only member of the B-vitamins fed, however, when riboflavin and pyridoxin, or these plus additional B vitamins are given in addition to thiamine, inositol is definitely lipotropic. Engel[3] also has shown that inositol exerts a lipotropic effect in the absence of biotin in the food. The results obtained in the present investigation are in good agreement with those of the above authors.

In order to control scattering of food, especially during the deficiency period, the food was made up as a concentrated aqueous solution containing insoluble material in suspension. This diet had the following composition:

Casein, vitamin free, Labco brand	120	g
Sucrose	1200	"
Salt mixture (Wesson)[4]	36	"
Cellu flour B*	12	"
Percomorph oil, 50%	0.5	cc
Water, distilled	960	"
Vitamin B-complex as given in text		

Experimental. White rats weighing 70-90 g were put in individual cages and fed daily 10-15 cc of the above diet, supplemented with 3 mg of thiamine chloride per 1200 g of sucrose. A 15 cc Folin and Wu pipet with a broken tip was used for measuring it. The amount given was so regulated that it was completely, or almost completely, consumed each day. After the animals were on this deficient diet for 3 weeks they were put on

TABLE I. Neutral Fat and Cholesterol Content of Liver, Body, and Skin.

Exp. No.	1		2		3		4		5		6		7			
Thiamine chloride	+		+		+		+		+		+		+			
Riboflavin			+		+		+		+		+		+			
Pyridoxin chloride			+		+		+		+		+		+			
Ca pantothenate			+		+		+		+		+		+			
Niacin			+		+		+		+		+		+			
Choline chloride, mg*																
Inositol mg*																
No. of rats	2	4	10	4	20	4	10	5	20	5	10	3	10	4	5	4
Daily avg gain wt, g	0.6	1.6	1.9	1.4	1.8	1.9	2.3	2.5	0.6	0.6	1.9	1.6	2.4	1.8	1.9	2.1
Avg liver wt, g	4.75	5.25	5.18	6.76	5.66	5.26	5.40	5.20	5.50	5.00	6.05	5.10	4.73	4.98	5.88	4.62
Liver neutral fat†	9.2	9.8	7.1	18.7	9.5	11.1	3.5	7.7	7.1	10.4	6.4	20.9	11.0	5.8	9.9	16.2	4.1
Liver cholesterol	.45	.56	.45	.73	.45	.54	.33	.74	.48	.61	.54	.82	.73	.48	.55	.55	.56
Body neutral fat†	5.5	7.7	8.3	6.2	8.5	6.6	6.7	9.8	9.8	6.0	6.3	8.8	8.7	9.4	8.5	8.9	9.9
Body cholesterol†	.18	.16	.19	.15	.19	.18	.18	.22	.22	.19	.21	.17	.17	.16	.18	.17	.18
Skin neutral fat†					14.8	15.2	21.3	21.3	13.2	14.7	17.0	15.6	20.4	17.2	17.0	21.3	
Skin cholesterol†					.21	.22	.18	.19	.27	.31	.17	.16	.14	.19	.18	.20	

* Mg per 10 g of sucrose in diet. † % wet weight. All animals in experiments 3, 4 and 5 received 0.5 γ of biotin per 10 g of sucrose in the diet during the supplemental feeding periods.

[1] Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, 1941, **139**, 485.

[2] Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, 1943, **148**, 275.

[3] Engel, R. W., *J. Nutrition*, 1942, **24**, 175.

[4] Wesson, L. G., *Science*, 1932, **75**, 339.

* Obtained from Chicago Dietetic Supply House, Chicago.

additional supplements for a period of 9-11 days, following which the animals were sacrificed for lipid analyses. These supplements were always given at the following levels, riboflavin 9 mg, pyridoxine hydrochloride 6 mg, calcium pantothenate 21 mg, niacin 21 mg, and biotin 60 γ per 1200 g of sucrose. Choline chloride and inositol were administered as recorded in the accompanying table.

The animals were rapidly exsanguinated by decapitation at the end of the supplemental feeding period. Their livers were removed, weighed, and after grinding in a mortar, analyzed individually for neutral fat and cholesterol. The intestinal tract was removed and the animal skinned, after which the neutral fat and cholesterol content of both the bodies and skins from each group was collectively determined. The analytical procedures have been described previously.[5,6]

From the experimental results, which are recorded in Table I, it will be seen that the lipotropic action of inositol in the absence of biotin from the diet is confirmed. The effect, however, is much less than one would expect from choline under the same conditions. The effect of choline plus inositol is greater than of either alone but the data are not sufficient to show whether they act synergistically or not. The effect on liver cholesterol is, on the whole, less than on the neutral fat. The small amount of biotin used in experiments 3, 4 and 5 did not apparently influence the lipotropic action of the inositol, neither did it increase the lipid content of the livers in the absence of inositol. These results are contrary to those obtained by McHenry and his associates[1] but it is possible that if we had used larger amounts of biotin, choline-resistant inositol-sensitive fatty livers might have been obtained.

The neutral fat content of the body and skin of those animals receiving both choline and inositol is greater than that of the corresponding animals receiving these individually. Whether this is due to an increased food intake, as suggested by the greater rate of growth of these animals, or to a stimulating effect on fat synthesis, cannot be stated at this time as food intake was not sufficiently accurately controlled to justify definite conclusions.

Summary. The lipotropic action of inositol in the presence of other members of the vitamin B-complex has been confirmed. The effect of inositol plus choline is considerably greater than that of either alone.

[5] Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1940, **25**, 1157.

[6] Forbes, J. C., *J. Nutrition*, 1941, **22**, 359.

BIOCHIMICA ET BIOPHYSICA ACTA

BBA 26304

METABOLISM OF *MYO*-INOSITOL IN RABBIT KIDNEY AND IN MAN

NORBERT FREINKEL, GABOR ANTONY*, HOLLIS R. WILLIAMS AND BERNARD R. LANDAU

Section of Endocrinology and Metabolism, Departments of Medicine and Biochemistry, Northwestern University Medical School, Chicago, Ill., Department of Medicine, Case-Western Reserve University School of Medicine, Cleveland, Ohio and Merck Institute for Therapeutic Research, Rahway, N.J. (U.S.A.)

(Received October 13th, 1969)

SUMMARY

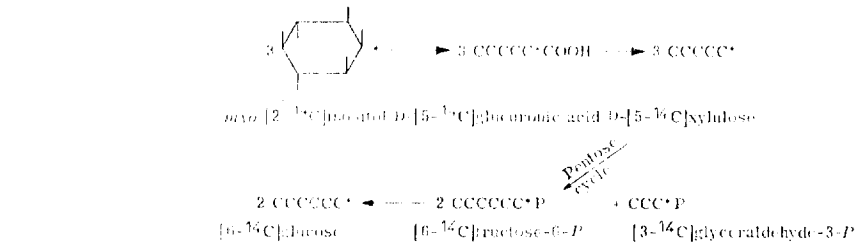
myo-[2-^{14}C]Inositol was incubated with slices of rabbit kidney cortex and was administered to a normal subject. Glucose and lactate, in the media from the incubations and in the blood of the subject, were isolated, degraded, and assayed for the ^{14}C distribution in each of their carbons. Distribution of ^{14}C in glycerol from the phospholipids in the slices was also determined. ^{14}C was primarily in C-1 and C-6 of glucose with greater activity always in C-6 than in C-1. ^{14}C was primarily in C-3 of the lactate and glycerol. These distributions are consistent with gluconeogenesis from *myo*-[^{14}C]-inositol in kidney proceeding *via* the glucuronic pathway and pentose cycle. This premise is strengthened by a comparison with the labeling patterns in glucose and lactate obtained on incubating kidney slices with [6-^{14}C]fructose.

INTRODUCTION

CHARALAMPOUS *et al.*[1] found that homogenates of kidney metabolized *myo*-inositol and they isolated an enzyme from kidney which converted *myo*-[2-^{14}C]inositol to D-[5-^{14}C]glucuronic acid. HOWARD AND ANDERSON[2] isolated D-[5-^{14}C]glucuronic acid from kidney slices after incubating them with *myo*-[2-^{14}C]inositol. Glucose is formed from *myo*-inositol by rabbit kidney slices (N. FREINKEL, unpublished observation) and this gluconeogenesis can occur from D-glucuronic acid by a well established sequence of reactions involving first the conversion of the D-glucuronic acid to D-xylulose and then subsequent metabolism of this pentose *via* the pentose cycle[3]. POSTERNAK and co-workers[4,5] administered *myo*-[2-^{14}C]inositol, *myo*-[2-^{3}H] inositol and *myo*-[6-^{3}H]inositol to phlorizin-treated rats and found distributions of ^{14}C and ^{3}H in glucose in urine in accord with metabolism by these reactions.

* Present address: Department of Medicine and Pediatrics, University of Vermont College of Medicine, Burlington, VT, U.S.A.

myo-[2-^{14}C]Inositol metabolized by these reactions would be expected to yield [6-^{14}C]glucose[6]:



Isomerization of the [3-^{14}C]glyceraldehyde 3-phosphate to [3-^{14}C]dihydroxyacetone 3-phosphate and then condensation of these triose phosphates, catalyzed by aldolase, should yield [1,6-^{14}C]glucose as would cleavage of the [6-^{14}C]fructose 6-phosphate to the triose phosphates, their isomerization and recombination.

ANDERSON AND COOTS[7] gave *myo*-[2-^{14}C]inositol to rats and 8-12 h post-injection found in each of three experiments about 80% of the ^{14}C in the glucose unit of liver and muscle glycogen in C-1 and C-6 of the unit. In two of these degradations, the activity in C-1 exceeded that in C-6. While extensive cleavage of [6-^{14}C]fructose 6-phosphate to [3-^{14}C]triose 3-phosphate and then resynthesis could approach equal labeling in C-1 and C-6, there is no good explanation, by presently accepted pathways, for greater activity in C-1 than C-6. Indeed, as HOWARD AND ANDERSON[8] noted, ^{14}C would have been predicted to be largely in C-6. Thus, glucose from glycogen of white blood cells incubated with D-[5-^{14}C]xylose contains ^{14}C almost exclusively in C-6 (ref. 8). HOWARD AND ANDERSON[8] have attributed the migration into C-1 to "secondary processes occurring during the long residence of the glucose in the kidney". In support of this they noted that P. A. WEINHOLD (unpublished data) found considerably higher activity in C-6 than C-1 of glucose from urine of phlorizin-treated rats given *myo*-[2-^{14}C]inositol. Glucose was assumed to remain in the kidney for a longer time in the normal than in the phlorizin-treated rat. However, HOWARD AND ANDERSON[8] further noted that there still was substantial activity in C-1 and that the mechnism by which this occurred remained to be elucidated. CHAROLLAIS AND POSTERNAK[1] found 32.6% of the ^{14}C in urinary glucose from phlorizin-treated rats given *myo*-[2-^{14}C]inositol in C-1 and C-2 of the glucose and 55.2% in C-6.

The distribution pattern in glucose observed by POSTERNAK, ANDERSON AND COOTS and WEINHOLD may be consequent at least in part to metabolism of the labeled *myo*-inositol in tissues other than kidney and perhaps to some extent from recycling of *myo*-inositol resynthesized from glucose[9]. We have degraded the glucose and lactate formed slices of rabbit kidney cortex from [2-^{14}C]*myo*inositol and compared the distributions with those obtained on incubating [6-^{14}C]fructose with slices and administering *myo*-[2-^{14}C]inositol to a normal subject. We have also examined the distribution of label in the glycerol of the phosphoglycerides formed during incubation of kidney slices with *myo*-[2-^{14}C]-inositol.

MATERIALS AND METHODS

Incubation of kidney slices with myo-[2-^{14}C]inositol

Slices (0.3–0.4-mm thickness) of the cortices of the kidney from rabbits were incubated in a modified Krebs Ringer bicarbonate buffer of composition 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.8 mM $CaCl_2$, 1.2 mM KH_2PO_4 and 25 mM $NaHCO_3$. The rabbits had either been fed a standard stock diet or fasted 24 h before killing. 450–500 mg of slices were incubated in each flask. The medium contained glucose at a concentration of 0, 35 or 120 mg per 100 ml. *myo*-[2-^{14}C]Inositol (purchased from Calbiochem, Lot No. 880435, specific activity 11.8 mC/mmole) was added at a concentration of 8.4 μg/ml.

Incubation was 1, 3 or 4 h. The slices were then removed and frozen in liquid N_2 and the media were then frozen. The media were subsequently thawed and chilled acetone was added in equal volume to aliquots from each medium on ice. The resulting suspensions were centrifuged and 300 μg of each supernatant was applied for chromatography (except for experiments designated 5 and 7 where the media were deproteinized by heat coagulation and 200 μg was applied). Chromatography was performed in two dimensions on Whatman No. 1 filter paper as described elsewhere[19]. The application site was overlaid with 20 μg of glucose. The first dimension was with ionophoresis at 4000 V for 30 min in pyridine–acetic acid–water (1:10:289, by vol.) at pH 3.7. The second dimension was with ascending chromatography in isopropanol–pyridine–acetic acid–water (8:8:1:4, by vol.). This clearly resolved lactic acid and the area containing the lactate was cut out and eluted with water. Carrier lactate was added and an aliquot of the solution was evaporated and then combusted to CO_2 to give the total ^{14}C activity in the solution. The lactate in the remainder of the solution was degraded[6] to give the activity in each of its three carbons. In the tables, to follow the activity of each of the carbons of lactate is given as a percentage of the sum of the activities in the three carbons which have been set equal to 100. Percent recovery recorded in the tables is the percentage of the activity in the solution as determined by combustion which this sum represents. Percent recoveries serve as a measure of the adequacy of the isolation and degradation. Recoveries were in general 90–105%.

Although the chromatography clearly resolved glucose and *myo*-inositol, specimens obtained following incubation disclosed some streaking in the glucose area. Accordingly glucose spots were eluted, concentrated and reapplied for ascending chromatography in third dimension in *n*-butanol–ethanol–water (52:30:18, by vol.). The glucose spots were then cut out and eluted with water, an aliquot combusted to CO_2 and the ^{14}C activity in the CO_2 determined. The remainder of the solution was then incubated with *Leuconostoc mesenteroides* to yield C-1 of glucose as CO_2, C-2 and C-3 as ethanol and C-4, C-5 and C-6 as lactate[6]. The ethanol and lactate were degraded as previously described to yield each of the carbons of glucose as CO_2 and the CO_2 was assayed for ^{14}C activity. Results recorded in the tables to follow are as for lactate except that the sum of the activities in the six carbons has been set equal to 100. Percent recoveries are the percent of ^{14}C in the solution recovered in the individual carbons of glucose. In general, recoveries were poor and because of this, for each of two samples, a glucosazone was made from an aliquot. The activity in the glucosazone was in good agreement with the sum of the activities in the individual

TABLE I

DISTRIBUTION OF ^{14}C IN GLUCOSE FORMED FROM *myo*-[2-^{14}C]INOSITOL BY RABBIT KIDNEY SLICES

Expt. No.	Conditions			^{14}C in glucose carbon						Recovery (%)
	Dietary state	Medium glucose (mg/100 ml)	Incubation period (h)	C-1	C-2	C-3	C-4	C-5	C-6	
1	Fed	35	1	40.9	3.1	1.7	2.3	1.8	50.2	80.4 (94.1)*
2	Fed	120	1	31.3	7.8	2.0	3.2	3.2	52.5	82.1
3	Fed	35	4	17.6	11.1	6.0	4.8	10.3	50.2	52.0
4**	Fed	120	3	25.5	←	———	11.4	———	→	Lost
5	Fast	35	1	24.8	7.4	2.3	2.9	3.9	58.7	73.4 (100.2)*
6	Fast	35	4	16.5	7.7	3.6	5.5	5.8	60.9	82.1

* Recovery in osazone in parentheses.

** Activities of C-1 and C-2–C-5 are recorded as the percent of the activity in the glucose obtained on combustion.

carbons. This is evidence that the activities attributed to the individual carbons of glucose were indeed of glucose and that a non-osazone forming radioactive compound, contaminating the sample, did not yield ^{14}C in the products of degradation. MUNTZ[11] has also noted the specificity of the degradation in the presence of contaminating substances.

In one experiment (Table I, Expt. 4) the glucose was converted to gluconic acid with glucose oxidase and the gluconic acid purified and degraded with HIO_4 to yield C-1 as CO_2, C-2–C-5 as formic acid and C-6 as formaldehyde and these products were assayed for ^{14}C (ref. 12). The evidence for the adequacy of the procedure was established by degradations of [1-^{14}C]glucose and [6-^{14}C]glucose.

Slices were homogenized in a glass homogenizer containing 4 ml of chloroform–methanol (2:1, by vol.). The homogenates were centrifuged, the volume adjusted to exactly 10 ml with chloroform–methanol (2:1, by vol.), and the phases separated with 2 ml of water[13]. The lipid extract was washed three additional times with "upper phase" (ref. 13) and partitioned into neutral lipids and phospholipids as previously described[14]. The phospholipids were subjected to mild alkaline hydrolysis and the water-soluble glycerylphosphoryl–base units isolated[15]. Glycerylphosphorylcholine and glycerylphosphorylethanolamine were eluted, pooled and then hydrolyzed for 24 h with 5 ml 2 M HCl after adding 20 μg of carrier glycerol. The hydrolysates were taken to dryness *in vacuo* to remove HCl. They were resuspended in water and then chromatographed two-dimensionally on Whatman No. 1 filter paper with phenol–water–acetic acid–ethanol (9:1:1:1.2, by vol.) descending followed by isopropanol–pyridine–acetic acid–water (1:8:1:4, by vol.) ascending. The spots were localized by radioautography and only two radioactive spots were discernible. One spot had the chromatographic and staining characteristics of glycerol reference markers and it was cut out, and designated as "glycerol". The second had the characteristics of glycerophosphate. To document its identity further, it was eluted and reapplied for ionophoresis at 4000 V for 30 min in pyridine–acetic acid–water (1:10:289, by vol.) at pH 3.7. It remained homogeneous and displayed the ionophoretic characteristics of glycerophosphate. It was therefore cut out and designated "glycerophosphate".

Carrier glycerol was added to an aqueous extract of the glycerol spots and the resulting solutions were incubated with *Aerobacter aerogenes*. This bacteria converts glycerol to lactate and the lactate was isolated and degraded and the resulting CO_2's

assayed for ^{14}C (ref. 6). One glycerophosphate sample was hydrolyzed by incubation with alkaline phosphatase. The hydrolyzate, after addition of carrier glycerol, was passed down a mixed-bed ion-exchange resin (Amberlite MB-3, Rohm and Haas Co., Philadelphia, Pa., U.S.A.) and the glycerol in the effluent was degraded using *A. aerogenes*.

Incubations of kidney slices with D-[6-^{14}C]*fructose*

In one experiment D-[6-^{14}C]fructose (117.6 μg/ml, 0.8 μC/ml purchased from Nuclear Research Corp., Orlando, Fla.) was the labeled substrate rather than *myo*-[2-^{14}C] inositol, but unlabeled *myo*-inositol was added at 8.4 μg/ml. Glucose was present at a concentration of 35 mg per 100 ml. Two flasks were incubated, one with slices of cortex from a fed rabbit, the other with slices from a fasted rabbit and the former incubation was for 1 h and the latter for 4 h. At the completion of incubation the media were heat coagulated and filtered. Analysis by a glucose oxidase method showed 9.8 mg/100 ml glucose remaining in the flask incubated for 1 h and 0.8 mg per 100 ml in that for 4 h.

A portion of the medium was chromatographed in two dimensions as described above. The lactate spot was then eluted and the lactate degraded and assayed. The remainder of the media was passed through a column packed with Amberlite MB-3. Effluent and aqueous washings from the column were evaporated to a small volume which was applied to Whatman 3 MM paper. Descending chromatography was performed using a *n*-butanol acetic acid water (4:1:5, by vol.) system with fructose and glucose guide spots. The area corresponding to glucose was eluted and the eluate was passed through another Amberlite MB-3 column. Carrier glucose was added and the solution degraded using *Leuconostoc mesenteroides*. A radioactive non-osazone forming contaminant was again present. This was considered to possibly be sorbitol, but treatment of an aliquot of the solution with sorbitol dehydrogenase did not yield radioactive fructose on chromatography.

Administration of myo-[2-^{14}C]*inositol to a normal volunteer*

myo-[2-^{14}C]Inositol (Calbiochem, Lot No. C-10361, specific activity 1 mC/mM), 50 μC, after autoclaving in saline was injected intravenously into a 23-year-old female volunteer (wt. 47.2 kg, height 5'3") who had been fasted overnight. 100 ml of blood was drawn at 1, 2 and 3 h after the injection. The blood was introduced into 700 ml of water and precipitated with 100 ml 0.15 M $Ba(OH)_2$ and 100 ml 5% $ZnSO_4$ (ref. 16). The supernatants from the samples obtained at 1 and 3 h were passed through a column of Duolite C-3 in the H^+ form and Duolite A-4 in the OH^- form (Diamond Alkali Co., Redwood City, Calif., U.S.A.). The effluent and aqueous washings of the column were evaporated to a small volume which was applied to Whatman 3MM paper and chromatographed in the butanol acetic acid water system. The glucose was isolated, degraded and the degradation products assayed for ^{14}C as described above.

The supernatant fluid from the 2-h period was evaporated to about 20 ml, acidified to pH 1 with 0.5 M H_2SO_4, carrier lactate was added and the solution was then continuously extracted with diethyl ether. The diethyl ether extract was neutralized with 1 M NaOH and evaporated. The lactate in the residue was purified on a celite column[17] and then degraded and assayed as described above.

RESULTS

The distribution of ^{14}C on the glucose formed from *myo*-[2-^{14}C]inositol (Table I) by the slices of kidney showed activity primarily in C-1 and C-6. There was 40–80% as much activity in C-1 as in C-6 in the 1-h experiments and 25–35% as much activity in the 4-h experiments. There was more activity in C-2–C-5 in the experiments of 4-h duration. No differences as a function of dietary state or the concentration of glucose in the medium are apparent.

The ^{14}C in the glucose from the blood of the normal subject given *myo*-[2-^{14}C]inositol (Table II) also was primary in C-1 and C-6. There was 70 and 85% as much activity in C-1 as in C-6 in the glucose from the blood drawn 1 and 3 h, respectively, after the *myo*-[2-^{14}C]inositol injection and greater randomization of ^{14}C into C-2–C-5 at 3 h than 1 h.

Lactate isolated from the medium after the incubations of Expts. 1 and 5 (Table III) contained ^{14}C primarily in C-3 while the lactate from the blood of the normal subject (drawn 2 h after the *myo*-[2-^{14}C]inositol administration) has 70% as much activity in C-1 as C-3.

Glycerol isolated from the phospholipid fraction contained activity primarily in C-3 (Table IV).

TABLE II

DISTRIBUTION OF ^{14}C IN BLOOD GLUCOSE FROM NORMAL SUBJECT ADMINISTERED *myo*-[2-^{14}C] INOSITOL

Time after injection (h)	*^{14}C in glucose carbon*						*Recovery (%)*
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	
1	35.0	4.0	1.5	2.0	5.0	51.9	94.8
3	35.8	5.2	1.0	4.8	7.8	41.8	100.3

TABLE III

DISTRIBUTION OF ^{14}C IN LACTATE FORMED FROM *myo*-[2-^{14}C]INOSITOL BY KIDNEY SLICES AND THE NORMAL SUBJECT

Source	*^{14}C in lactate carbon*			*Recovery (%)*
	C-1	*C-2*	*C-3*	
Expt. 1	6.0	0.6	87.4	87.6
Expt. 5	5.4	0.4	88.8	98.0
Normal subject	36.1	12.1	51.8	100.2

TABLE IV

DISTRIBUTION OF ^{14}C IN GLYCEROL FORMED FROM *myo*-[2-^{14}C]INOSITOL BY RABBIT KIDNEY SLICES

Expt. No.	*^{14}C in glycerol carbon*			*Recovery (%)*
	C-1	*C-2*	*C-3*	
2	34.4	6.3	59.3	100.0
3	5.0	0.9	84.3	112.0[**]
4	15.3	9.3	75.4	92.6
7*	31.7	7.0	61.3	96.3

* In Expt. 7 only glycerol was determined. Slices were from fed rabbits, incubation was for 3 h and was done in a medium not prepared with glucose.

** Glycerol was isolated from the total phosphoglyceride by treatment with alkaline phosphatase.

TABLE V

INCORPORATION OF CARBON IN GLUCOSE AND LACTATE FORMED FROM D-[6-^{14}C]FRUCTOSE BY RABBIT KIDNEY SLICES

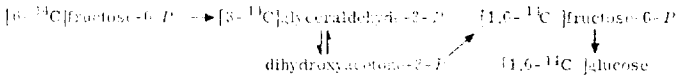
Expt. no.	Period of incubation (hr.)	^{14}C in glucose carbon						Recovery (%)	^{14}C in lactate carbon			Recovery (%)
		C-1	C-2	C-3	C-4	C-5	C-6		C-1	C-2	C-3	
1	1	19.3	4.6	0.7	2.1	1.1	72.2	66.2 (101.4)*	0.0	4.8	95.2	101.9
1-Acet†	4	17.2	6.6	3.0	2.9	3.3	67.0	16.7 (80.4)*	10.4	9.4	80.2	98.5

* Recovered in osazone in parentheses.

In the incubations of [6-^{14}C]fructose with rabbit slices (Table V) there was one-fourth as much ^{14}C in C-1 as C-6 and again ^{14}C was primarily in these carbons. Incorporation of ^{14}C in lactate was primarily in C-3.

DISCUSSION

In no instance have we observed a greater activity in C-1 than C-6 of glucose as was reported by ANDERSON AND COOTS[7]. The distributions in glucose we observe are in accord with the metabolism *via* the glucuronic acid pathway and pentose cycle. These results document *in vitro* what has been presumed to occur in the kidney from studies *in vivo*. In the slice experiment (Table I) the incorporation into C-1 can be attributed to both the equilibration of the [3-^{14}C]glyceraldehyde 3-phosphate formed *via* these pathways (see INTRODUCTION) with dihydroxyacetone 3-phosphate and then formation of glucose, and the cleavage of fructose 6-phosphate formed from the *myo*-[2-^{14}C]inositol into the triose phosphates, their equilibration and then formation of glucose, *i.e.*:



The significance of the lesser incorporation into C-1 relative to C-6 after 4 h of incubation than after the earlier time periods is uncertain because of the limited number of experiments performed. The extent of incorporation into C-1 compared to C-6 would be dependent upon the rate of isomerization of glyceraldehyde 3-phosphate to dihydroxyacetone 3-phosphate relative to the rate of formation of the glucose and these rates could have changed with time. The greater activity in C-2–C-5 at 4 h than at the earlier hours is in accord with increasing randomization with time of the ^{14}C into oxaloacetate and pyruvate *via* the reactions of the Krebs cycle and the synthesis of glucose from these compounds. The distribution of ^{14}C in lactate (Table II) is also in accord with such metabolism with the ^{14}C in C-3 reflecting formation from [3-^{14}C]glyceraldehyde 3-phosphate and the ^{14}C in C-1 and C-2 reflecting randomization *via* the reactions of the Krebs cycle. Since *myo*-inositol can be synthesized from glucose by kidney slices[18], the ^{14}C distributions could conceivably be consequent in some degree to the metabolism of ^{14}C labeled *myo*-inositol formed from the ^{14}C labeled glucose in turn formed from the *myo*-[2-^{14}C]inositol added as labeled substrate. This contribution was almost certainly negligible in consideration of the dilution of the ^{14}C of the *myo*-[2-^{14}C]inositol during this recycling and the limited amount of conversion of glucose to *myo*-inositol[18].

The distributions of ^{14}C in the glucose from the blood of the subject given *myo*

[2-^{14}C]inositol are similar to those obtained using the kidney slices, though there perhaps was a tendency to greater incorporation into C-1 relative to C-6 by the subject. Most probably the glucose in the blood was formed primarily from *myo*-[2-^{14}C]-inositol without lactate as an intermediate. If the glucose has been formed from [3-^{14}C]lactate one would expect glucose with ^{14}C extensively in C-2 C-5 in accord with the metabolism of the carbons of lactate in the Krebs cycle prior to their conversion to gluco-c[19].

C-1 to C-6 ratios when [6-^{14}C]fructose was substrate were in general lower than when *myo*-[2-^{14}C]inositol was substrate. If in kidney the [6-^{14}C]fructose was phosphorylated to [6-^{14}C]fructose 6-phosphate rather than [6-^{14}C]fructose 1-phosphate this would be expected since incorporation into C-1 from the [6-^{14}C]fructose could then only occur through cleavage to the triose phosphates and then resynthesis. The data would indicate that much of [6-^{14}C]fructose was phosphorylated to [6-^{14}C]fructose 6-phosphate since each molecule of [6-^{14}C]fructose 1-phosphate would yield in its further metabolism a molecule of [3-^{14}C]glyceraldehyde 3-phosphate and this would then favor increased incorporation into C-1. In accord with this the activity in the rat of the enzyme fructokinase, catalyzing the formation of fructose 1-phosphate, is several-fold lower in kidney than in liver[20]. In liver where the fructose 1-phosphate pathway is so active, C-1 of glucose formed from [6-^{14}C]fructose does have 70–80% of the activity of C-6 (ref. 11). This is also the case for blood glucose when [6-^{14}C]-fructose is administered to man (J. MARSHALL, J. CRAIG, K. HOSTETLER AND B. R. LANDAU, unpublished observations).

The considerable activity in C-1 in two degradations of glycerol formed by kidney slices and in the blood lactate from the subject given *myo*-[2-^{14}C]inositol remain unexplained. The glycerols would be expected to reflect the distribution in glyceraldehyde 3-phosphate and hence have activity primarily in C-3 and the lactate should be expected to have a distribution similar to that seen in the experiments *in vitro*.

Note added in proof (Received December 18th, 1969)

Since submission of this paper Hankes *et al.*[21] have reported a study of the metabolism of myoinositol in humans. In three normal subjects three nonpentosuric kindred of subjects with pentosuria, two hours after administration of myo-[2-^{14}C]inositol 31±2% and 48±3% of the ^{14}C in blood glucose was recovered in C-1 and C-6 respectively, a distribution similar to that in our one subject.

ACKNOWLEDGMENTS

This study was supported by Research Grants AM-10627 and AM-10699 and by Training Grant AM-5071 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service, Bethesda, Md., U.S.A.

REFERENCES

1 E. C. CHARALAMPOUS, S. BUMILLER AND S. GRAHAM, *J. Am. Chem. Soc.*, 80 (1958) 2022.
2 C. F. HOWARD, JR. AND L. ANDERSON, *Arch. Biochem. Biophys.*, 118 (1967) 332.
3 J. J. BURNS, in D. M. GREENBERG, *Metabolic Pathways*, Academic Press, New York, 3rd Ed., 1967, p. 105.
4 E. CHAROLLAIS AND T. POSTERNAK, *Helv. Chim. Acta*, 48 (1965) 280.
5 T. POSTERNAK, *The Cyclitols*, Holden Day, San Francisco, 1965, p. 329.
6 B. R. LANDAU, G. E. BARTSCH AND H. R. WILLIAMS, *J. Biol. Chem.*, 241 (1966) 750.
7 L. ANDERSEN AND R. H. COOTS, *Biochim. Biophys. Acta*, 28 (1958) 666.
8 R. STERNHOLM AND E. P. NOLLE, *J. Biol. Chem.*, 236 (1960) 914.
9 N. FREINKEL AND R. M. C. DAWSON, *Biochem. J.*, 81 (1961) 250.
10 N. FREINKEL, A. K. COHEN, R. A. ARKY AND A. L. FOSTER, *J. Clin. Endocrinol. Metab.*, 25 (1965) 756.
11 J. A. MUNTZ, *J. Biol. Chem.*, 243 (1968) 2788.
12 L. W. WHITE AND B. R. LANDAU, *J. Clin. Invest.*, 44 (1965) 1200.
13 J. FOLCH, M. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
14 N. FREINKEL, A. D. LITTLE AND F. C. SALE, *Nature*, 191 (1961) 804.
15 R. M. C. DAWSON, N. HEMINGTON AND J. B. DAVENPORT, *Biochem. J.*, 84 (1962) 497.
16 M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19.
17 E. H. SWIN AND L. O. KRAMPITZ, *J. Bacteriol.*, 67 (1954) 419.
18 G. HAUSER AND V. N. FINELLI, *J. Biol. Chem.*, 238 (1963) 3224.
19 K. Y. HOSTETLER, H. R. WILLIAMS, W. W. SHREEVE AND B. R. LANDAU, *J. Biol. Chem.*, 244 (1969) 2075.
20 R. C. ADELMAN, F. J. BALLARD AND S. WEINHOUSE, *J. Biol. Chem.*, 242 (1967) 3360.
21 L. V. HANKES, W. M. POLIBZER, O. TOUSTER, AND L. ANDERSON, *Ann. N.Y. Acad. Sci.*, 165 (1969) 504.

J. Biol. Chem. 139:485, 1941

INOSITOL: A LIPOTROPIC FACTOR

Sirs:

It was reported by us last year that feeding a beef liver fraction to rats causes the production of acutely fatty livers containing large amounts of cholesterol. The production of these fatty livers is only slightly affected by giving choline but is completely prevented by the simultaneous administration of lipocaic. Using a procedure similar to that for the preparation of lipocaic, we have prepared extracts from liver, pancreas, kidney, muscle, wheat germ, yeast, and rice polishings and have found that these are all effective in preventing this type of fatty liver in rats. It has been reported by us that the same kind of fatty liver can be produced by supplying rats with biotin in conjunction with thiamine, riboflavin, pantothenic acid, and pyridoxine.

In studies on the prevention of the "biotin" type of fatty liver in rats we have fed inositol. This substance prevents the development of the acutely fatty liver and the accumulation of cholesterol in the liver. The prevention of the fatty liver has been secured in three series of animals, in each of which groups of ten rats have been employed. In these experiments on rats the action of inositol thus resembles that of lipocaic.

School of Hygiene
University of Toronto
Toronto, Canada

GERTRUDE GAVIN
E. W. MCHENRY

Received for publication, April 21, 1941

J. Biol.Chem.141:619 -625, 1941

THE EFFECTS OF BIOTIN UPON FAT SYNTHESIS AND METABOLISM

By GERTRUDE GAVIN and E. W. McHENRY

(From the School of Hygiene, University of Toronto, Toronto, Canada)

(Received for publication, August 2, 1941)

In previous papers (1, 2) we reported that the administration to rats of an alcohol-soluble fraction of beef liver caused markedly fatty livers, characterized by a high content of cholesterol. Coincidentally there was a gain in body fat and body weight. The fatty livers thus produced differed from the thiamine type of fatty livers in that they were not prevented by supplying choline but were prevented by feeding lipocaic. In a continuation of this work we have found that biotin exerted an action similar to that of the crude liver fraction.

Methods

Rats were employed as test animals. The strain, age, and care were the same as previously reported (3). Basal Diet 1 (3) was used throughout. To deplete the rats of their stores of B vitamins and of fat, they were fed only the basal diet for 3 weeks. At the end of this period body weight and body fat had definitely diminished. During the following week various combinations of supplements were administered to different groups. Thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, choline, and the crystalline biotin solutions were given by subcutaneous injection. Supplements other than those previously mentioned were mixed with the food. Vitamin and choline supplements were given in the following amounts per rat per day, unless otherwise indicated: thiamine hydrochloride (Merck) 25 γ, riboflavin (Merck) 20 γ, pyridoxine (Merck) 20 γ, calcium pantothenate (Merck) 100 γ, nicotinic acid (Eastman Kodak Company) 100 γ, choline hydrochloride (British Drug Houses), calculated as choline base, 10 mg., biotin (kindly donated by S. M. A. Corpora-

tion) 5 γ, lipocaic (generously furnished by Eli Lilly and Company) 300 mg.

The rats were killed by stunning, the livers removed, and total crude fatty acids in the livers and bodies were determined by methods previously published (3, 4). By means of the Schoenheimer and Sperry procedure (5), the total cholesterol in the crude fatty acid fraction of the liver and body was determined. All reported results are averages for groups of ten rats, except for one group in Series 4.

Effect of Biotin upon Fat Synthesis

Series 1—A liver fraction, similar to the one used by us (2), has served as a source of biotin in other laboratories (6–8). It seemed advisable to ascertain whether biotin would produce a fatty liver, not preventable by choline, resembling that caused by the liver fraction. A solution of biotin, prepared in this laboratory from the liver fraction by the method of György, Kuhn, and Lederer (7), and several commercial solutions have been tested. The total crude fatty acids in the liver and body at the end of the experiment are given in Table I. The biotin solutions exerted an effect upon liver fat similar to that of the liver fraction.

Series 2—Preliminary experiments showed that biotin would not cause fatty livers unless other B vitamins were supplied. The effect of the known B vitamins upon the activity of biotin was investigated. Acutely fatty livers were not produced unless thiamine, riboflavin, pantothenic acid, and pyridoxine were supplied with biotin. In the absence of thiamine there was no evidence of fat synthesis. An additive effect of the various B vitamins was also evident. The total crude fatty acids in the liver and body are given in Table II.

Series 3—The rate of development of fatty livers in rats fed biotin was investigated. The results are given in Table I. Data regarding liver and body cholesterol are also included.

Series 4—In the series reported above the biotin solutions were all comparatively crude. In addition to the two mentioned, biotin solutions kindly contributed by Merck and Company, Inc., and by The Fleischmann Laboratories showed similar activity. In all these cases it might be said that the results were not due to biotin but to an impurity contained in the preparations. Through the

TABLE I

Effect of Biotin upon Fat and Cholesterol Metabolism

Series No.	Supplements	Total crude fatty acids		Total cholesterol	
		Liver	Body	Liver	Body
		per cent	per cent	per cent	per cent
1	None, 3 wks. depletion	2.4	1.7		
	All isolated B vitamins, choline	9.5	6.5		
	Same + liver fraction	17.2	7.7		
	" + biotin (S. M. A.)	17.8	5.9		
	" + " (School of Hygiene)	17.9	6.7		
3	None, 3 wks. depletion	4.6	1.9	0.32	0.15
	All isolated B vitamins, choline, biotin				
	24 hrs.	4.0	2.4	0.22	0.18
	48 "	7.1	3.0	0.34	0.19
	72 "	14.1	4.4	0.50	0.26
	5 days	18.3	6.1	0.80	0.26
	7 "	17.9	7.2	0.93	0.25
4	None, 3 wks. depletion	3.3	1.7	0.38	0.23
	All isolated B vitamins, choline	11.2	7.7	0.71	0.30
	Same + biotin (S. M. A.)	19.3	8.2	1.08	0.27
	" + " (crystalline)	17.8	7.3	1.25	0.28
5	All isolated B vitamins, choline	13.2	6.3	0.64	0.29
	Same + biotin (S. M. A.)	13.5	6.5	0.67	0.25
6	None, 3 wks. depletion	3.6	1.9	0.16	0.12
	All isolated B vitamins, choline	6.7	5.7	0.35	0.22
	Same + lipocaic	4.5	6.3	0.20	0.17
	" + biotin	15.6	5.9	0.79	0.16
	" + " + lipocaic	3.7	7.8	0.10	0.18

TABLE II

Effect of B Vitamins upon Fat Synthesis by Biotin in Series 2

Biotin		+		+	+	+	+	+	+
Thiamine		+	+		+	+	+	+	+
Riboflavin		+	+	+		+	+	+	+
Ca pantothenate		+	+	+	+		+	+	+
Pyridoxine		+	+	+	+	+		+	+
Nicotinic acid		+	+	+	+	+	+		+
Choline		+	+	+	+	+	+	+	
Total crude fatty acids									
Liver, %	3.3	18.3	10.7	2.5	5.2	8.6	11.4	21.6	25.5
Body, %	1.7	5.6	6.9	1.8	3.6	5.7	5.8	6.1	5.0

courtesy of Dr. du Vigneaud we have been able to test the activity of crystalline biotin. A solution of biotin methyl ester was supplied to us by Dr. du Vigneaud. Because of the limited amount available it was necessary to use only five animals. The biotin solution was injected subcutaneously with a dosage of 5 γ per rat per day. It will be observed that the crystalline material had an effect similar to that of the crude solutions.

Series 5—It has been reported that raw egg white will inactivate biotin *in vivo* (9). In this series a basal diet consisting of agar 2, salt mixture 4, sucrose 84, cod liver oil concentrate 0.015 parts was used. Each rat received, in addition, 4 cc. of raw egg white per day mixed with the basal diet. Under these conditions the biotin

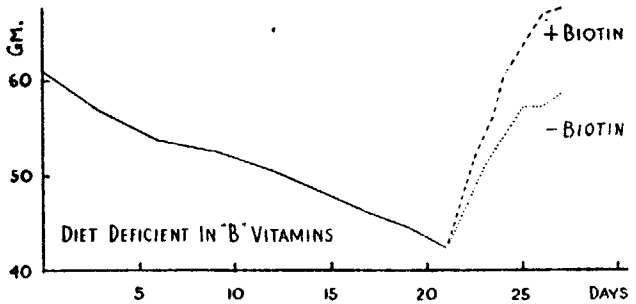


FIG. 1. The effect of biotin upon the body weight. During the supplement period thiamine, choline, riboflavin, pyridoxine, and Ca pantothenate were administered.

solution did not cause an increase in liver fat. We regard this as further evidence that biotin is the active agent in causing this type of fatty liver.

Series 6—It has been reported that lipocaic would prevent the development of fatty livers when the beef liver fraction was given (2). Lipocaic was equally effective in preventing the development of fatty livers when biotin was administered, as is shown in this series.

Biotin also resembled the active factor in the liver fraction by causing an increase in body weight, as shown in Fig. 1. A similar liver fraction has been employed as a source of factor W (10). It seems likely that the effect, ascribed to factor W, of causing weight increases might have been due to biotin, acting in conjunction with the other B vitamins. Other workers (9, 11) have noted the effect of biotin in causing an increase in the body weight of rats.

Effect of Biotin upon Cholesterol Metabolism

In Series 3, when biotin was given, the increase in cholesterol in the liver paralleled the increase in liver fat. Crystalline biotin, as well as the crude biotin solutions, caused the amount of cholesterol in the liver to be greater than in the control group receiving all the isolated B vitamins and choline, as shown in Series 4. Further proof that biotin is concerned in cholesterol metabolism is offered in Series 5, since raw egg white, given with biotin, was able to prevent not only an increase in liver fat but also an increase in liver cholesterol. Lipocaic, which prevented the increase in liver fat caused by biotin, maintained the liver cholesterol at a low level, as shown in Series 6.

DISCUSSION

Evidence has been presented which indicates that biotin is the active factor, in the alcohol-soluble fraction of beef liver, which causes the amount of fat and cholesterol in the livers of rats to be increased. Final proof that the beef liver fraction owes its activity in producing fatty livers to biotin could only be supplied by the isolation of biotin from the fraction. The following points of evidence are cited to indicate that our assumption is valid: (1) Crude solutions of biotin from several sources exhibited the same physiological action as did the liver fraction. (2) The activity of the crude biotin solutions was obliterated by the simultaneous feeding of egg white. (3) Crystalline biotin had the same physiological action as had the crude solutions of biotin, and as was shown by the liver fraction in producing acutely fatty livers containing large amounts of cholesterol.

Simultaneous administration of lipocaic prevented the increased fat and cholesterol in the liver caused by biotin, while choline was ineffective in this respect. Choline was able to prevent the development of the thiamine type of fatty liver but not the type caused by liver fraction or by biotin. We shall refer to this latter kind of fatty liver as the biotin type to distinguish it from that caused by thiamine. Previous reports have shown that the production of biotin fatty livers can be prevented by lipocaic-like

extracts made from wheat germ and other sources (12) and also by inositol (13).

Biotin produced an acutely fatty liver in a comparatively short time. A significant increase was observed in 24 hours and the maximal level of liver fat was secured in 5 days. It should be made clear that the production of these fatty livers was not due simply to withdrawal of fat from the body. Coincident with the development of fatty livers there was a marked increase in the amount of body fat. In Series 2 the quantity of body fat was increased 2.5 times in 5 days, owing entirely to synthesis, since no fat was furnished in the diet. It seemed possible that the production of the fatty liver by biotin was due to accumulation of cholesterol in the liver. This may be true, but the increase in liver cholesterol did not occur prior to the production of the fatty liver. Biotin did not increase the amount of cholesterol synthesis, but did augment the quantity of cholesterol in the liver.

Engel reported (14) that pantothenic acid caused the same type of fatty liver as that produced by the beef liver fraction. Our results show that choline does not completely prevent the development of fatty livers when thiamine, riboflavin, pyridoxine, and pantothenic acid are supplied. The liver does not, then, contain the large amounts of cholesterol evident when biotin is given. The amount of liver fat can be augmented by giving pantothenic acid in conjunction with thiamine, riboflavin, and pyridoxine, but the amount is much further increased by also supplying biotin. The effect of biotin is not secured unless thiamine, riboflavin, pyridoxine, and pantothenic acid are also furnished. The combined action of these five vitamins is necessary to secure the biotin fatty liver, which completely resembles that produced by the liver fraction.

SUMMARY

Impure solutions of biotin from several sources and pure biotin, given to rats in conjunction with thiamine, riboflavin, pyridoxine, and pantothenic acid, caused fatty livers similar to those produced by feeding a fraction from beef liver. The fatty livers were characterized by a high content of cholesterol. The effect of biotin was prevented by simultaneously feeding egg white, lipocaic, or inositol. It is concluded that beef liver fraction owes its activity

to its content of biotin. Biotin had, also, an additive effect upon body weight, similar to that ascribed to factor W.

This investigation has been assisted by a grant from the Division of Natural Sciences of the Rockefeller Foundation.

BIBLIOGRAPHY

1. McHenry, E. W., and Gavin, G., *Science*, **91**, 171 (1940).
2. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **134**, 683 (1940).
3. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940).
4. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **125**, 653 (1938).
5. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
6. Lease, J. G., and Parsons, H. T., *Biochem. J.*, **28**, 2109 (1934).
7. György, P., Kuhn, R., and Lederer, E., *J. Biol. Chem.*, **131**, 745 (1939).
8. György, P., Rose, C. S., Hofmann, K., Melville, D. B., and du Vigneaud, V., *Science*, **92**, 609 (1940).
9. György, P., Rose, C. S., Eakin, R. C., Snell, E. E., and Williams, R. J., *Science*, **93**, 477 (1941).
10. Elvehjem, C. A., Koehn, C. J., Jr., and Oleson, J. J., *J. Biol. Chem.*, **115**, 707 (1936).
11. du Vigneaud, V., Melville, D. B., György, P., and Rose, C. S., *Science*, **92**, 62 (1940).
12. McHenry, E. W., and Gavin, G., *Canad. Chem. and Process Ind.*, **24**, 640 (1940).
13. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 485 (1941).
14. Engel, R. W., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **140**, p. xxxvii (1941).

J. Biol. Chem. 148:275-279;1943

COMPARISON OF THE LIPOTROPIC EFFECTS OF CHOLINE, INOSITOL, AND LIPOCAIC IN RATS

By GERTRUDE GAVIN,* JEAN M. PATTERSON, AND E. W. McHENRY

(From the School of Hygiene, University of Toronto, Toronto, Canada)

(Received for publication, January 29, 1943)

In a preliminary communication (1) we reported that inositol would prevent the development of that type of fatty liver, characterized by a high content of cholesterol, which is produced in rats by the administration of biotin. The lipotropic action of inositol has been studied with other types of fatty livers and has been compared with the effects of two other lipotropic agents, choline and lipocaic.

Methods

Rats have been employed as test animals. The strain, age, and care have been previously described (2). To diminish the stores of B vitamins and of fat, the animals were fed Diet 1 for 3 weeks. During the 4th week the diet was varied as indicated for each series, the composition of the diets being given in Table I. Vitamin supplements were administered during the 4th week in the quantities previously used (4); the amounts of choline, lipocaic, and inositol used in each series are given in Tables II and III. Estimations of total crude fatty acids and of cholesterol were made by methods previously reported (2, 4). Results of these determinations are given as averages for groups of ten animals.

EXPERIMENTAL

Series A— This series was planned to compare the effects of choline, lipocaic, and inositol upon fatty livers caused by biotin. Diet 1 was fed throughout the experiment. During the supplemental period all animals received thiamine, riboflavin, pyridoxine, nicotinic acid, and calcium pantothenate; choline, biotin, inositol, and lipocaic were administered as indicated in Table II.

Series B— Since the results of Series A had indicated that inositol was concerned with cholesterol metabolism, it seemed advisable to test its effect upon rats fed cholesterol in a fat-free diet. Diet 1 was given during the depletion period and Diet 2 during the 4th week with thiamine, riboflavin, pyridoxine, nicotinic acid, and calcium pantothenate supplied to all animals. In those groups which received choline, an increased amount (30 mg. per rat per day) was administered, since Best and Ridout (5) had

* Deceased, November 9, 1942.

shown that large doses of choline would partially prevent the production of fatty livers in rats fed cholesterol.

TABLE I

Composition of Diets

Constituent	Diet 1	Diet 2	Diet 3
Casein, Labco, vitamin-free	10	10	10
Agar	2	2	2
Salt mixture (Steenbock-Nelson Salts 40 (3))	4	4	4
Sucrose	84	82	52
Cholesterol	0	2	2
Corn oil (Mazola)	0	0	30
Cod liver oil concentrate (Ayerst, McKenna, and Harrison)	0.015	0.015	0.015

TABLE II

Averages for Groups of Ten Rats Obtained during 4th Week of Experiment

Series	Basal diet No.	Special supplements	Crude fatty acids		Cholesterol	
			Liver	Body	Liver	Body
			per cent	*per cent*	*mg.*	*mg.*
A	1	None	15.3	5.6	19	95
		10 mg. choline	6.7	5.7	12	102
		5 γ biotin, 10 mg. choline	15.6	5.9	37	93
		5 " " 10 " " 200 mg. lipocaic	3.7	7.8	4	121
		5 " " 10 " " 2 mg. inositol	6.3	6.8	13	102
B	2	None	24.6	6.5	69	243
		30 mg. choline	9.1	7.3	37	279
		300 " lipocaic	13.8	6.0	36	254
		10 " inositol	13.3	6.9	27	298
		30 " choline, 300 mg. lipocaic	7.3	6.1	30	225
		30 " " 10 " inositol	3.9	7.1	18	264
C	3	None	26.0	9.5	72	205
		30 mg. choline	11.7	14.9	93	316
		300 " lipocaic	26.0	10.9	100	276
		10 " inositol	17.1	10.6	56	264
		30 " choline, 300 mg. lipocaic	8.7	13.6	71	215
		30 " " 10 " inositol	9.1	14.1	72	281
D	1	25 γ thiamine	10.2	4.3	15	142
		25 " " 10 mg. choline	3.1	4.2	8	132
		25 " " 10 " inositol	9.2	4.9	13	164
		25 " " 10 " choline, 10 mg. inositol	2.9	4.2	7	138

Series C In this case a high fat diet (No. 3) was used during the supplemental period, so that a comparison could be made with Series B, in which a diet practically devoid of fat was employed. Otherwise, the treatment of the animals was the same as in Series B.

Series D It has been reported previously from this laboratory that choline will prevent fatty livers caused by thiamine (6). This series deals with the effect of inositol upon this type of fatty liver. Diet 1 was used throughout the experiment; thiamine was the only B vitamin supplement.

Series E—As Series D had indicated that inositol had no appreciable effect upon thiamine fatty livers, its action when administered with several other B vitamins was investigated. Diet 1 was employed during all 4 weeks; the supplements used and the results obtained are given in Table III.

TABLE III

Effect of Inositol in Prevention of Fatty Livers with Various B Vitamins

Series E on basal Diet 1.

Special supplements										
Thiamine		+	+	+	+	+	+	+	+	+
Riboflavin		+	+	+	+	+	+	+	+	+
Pyridoxine		+	+	+	+	+	+	+	+	+
Nicotinic acid						+	+	+	+	+
Pantothenic acid				+	+			+	+	+
Inositol, 10 mg.			+		+		+		+	
Choline, 10 "										+
Crude fatty acids										
Liver, %	2.9	20.4	12.2	22.9	18.5	15.7	9.4	25.9	16.5	8.4
Body, %	2.0	5.4	5.9	6.4	6.7	5.1	4.9	6.8	7.3	6.8

DISCUSSION

While choline has little effect in preventing biotin fatty livers, as has been reported previously (4), both lipocaic and inositol have definite effects in preventing increases in both fatty acids and total cholesterol in the liver. It should be noted that the dosage of inositol used in Series A was small; in similar experiments amounts of 5 to 10 mg. were found to be as effective as 200 mg. of the lipocaic preparation.

When fatty livers are produced by feeding a fat-free diet containing cholesterol, choline, lipocaic, and inositol all have lipotropic action, not only with regard to fatty acids but also with regard to cholesterol. Under these conditions lipocaic or inositol is more effective when fed with choline than when supplied alone; this is particularly true in the case of inositol.

In Series A and B an increase in fat was obtained by synthesis. In Series C a comparison was made with animals receiving a high fat diet, and fatty livers were produced by feeding cholesterol. While choline and inositol showed lipotropic action, lipocaic appeared to be entirely ineffective.

This observation was confirmed in three other experiments. The lipocaic, which was kindly supplied by Dr. Lester Dragstedt, was a sample of material that had been effective in depancreatized dogs; its potency for rats was proved by the prevention of biotin fatty livers.

While inositol is without obvious effect on thiamine fatty livers, the addition of other B vitamins as supplements enabled inositol to exert appreciable lipotropic action. The dosages of choline and inositol were the same in both Series D and E. It is noteworthy that neither supplement maintained liver fat at a normal level in Series E. Apparently the addition of other B vitamins diminishes the effect of choline but makes possible a response with inositol.

It has been pointed out previously from these laboratories (7) that there are several different types of fatty livers. We have little information as to how these livers differ in composition, but they can be distinguished in two ways: causative agents, and response to lipotropic factors. In describing the action of a lipotropic substance it is essential, in the light of present knowledge, to give the method of production of the particular fatty liver used. Reports of inactivity of lipocaic in rats were due to failure to use a suitable type of fatty liver. Even choline has no appreciable effect in preventing biotin fatty livers.

A previous, preliminary report by two of us (1) stated that either inositol or lipocaic could be used for the prevention of biotin fatty livers. The impression could have been secured easily from this report that lipocaic owed its activity to inositol; at that time this possibility seemed very likely to us. The data now reported show that lipocaic is ineffective with one type of fatty liver in rats, while inositol is active. This observation provides a means of distinguishing between inositol and lipocaic as lipotropic agents and it appears that lipocaic may contain a factor other than choline (or choline precursors) or inositol. However, all lipocaic preparations examined in these laboratories contained appreciable amounts of inositol. It may be that the inositol is present in a compound from which it is set free only under certain conditions. Available evidence is not sufficient to permit a decision as to whether lipocaic owes its activity to inositol or to an unknown constituent.

SUMMARY

A comparison of the lipotropic effects of choline, lipocaic, and inositol has been made with various types of fatty livers caused by diet in rats. Choline is effective for thiamine fatty livers, and partially effective with cholesterol fatty livers, but shows little activity with biotin fatty livers. Against this last type both inositol and lipocaic are active. Lipocaic apparently differs from inositol in being ineffective against fatty livers caused by feeding cholesterol with a high fat diet. Inositol shows no activity with thiamine fatty livers; the addition of other B vitamins permits inositol to be lipotropic.

This investigation has been assisted by a grant from the Division of Natural Sciences of the Rockefeller Foundation.

BIBLIOGRAPHY

1. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 485 (1941).
2. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940).
3. Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, **56**, 362 (1923).
4. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **141**, 619 (1941).
5. Best, C. H., and Ridout, J. H., *J. Physiol.*, **86**, 343 (1936).
6. McHenry, E. W., *J. Physiol.*, **89**, 287 (1937).
7. McHenry, E. W., in Biological symposia, Lancaster, **5**, 177 (1941).

THE FATE OF INOSITE ADMINISTERED TO DOGS.

By ISIDOR GREENWALD and MORRIS L. WEISS.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

(Received for publication, May 7, 1917.)

In a recent publication Anderson[1] has reviewed the literature on the utilization of inosite by animals and has reported the results of his own experiments. All investigators who have worked with inosite have found that it is not readily utilized by animals. After administration by mouth, a considerable amount of inosite may disappear but, after subcutaneous administration, most of it is found in the urine. The destruction of inosite given by mouth has generally been ascribed to the action of the intestinal flora. In man, Anderson found that of 0.5 gm. of inosite per kilo of body weight given by mouth, only 9 per cent was found in the urine and none in the feces. In the dog, however, with doses of 2 gm. per kilo, apparently very little was absorbed from the intestine. A large part of the inosite administered could be recovered from the feces but only a small amount was found in the urine. Ingestion of inosite did not raise the respiratory quotient and Anderson concluded that "inosite is not utilized to any extent by the dog."

At the time Anderson's experiments were published we had already been engaged with an investigation of the same subject. Our first experiments were planned to ascertain whether or not inosite was, physiologically, related to the carbohydrates. Previous work had indicated that it was not. Külz[2] and Mayer[3] had failed to observe a formation of glycogen from inosite. It is true that Mayer had found a small amount of lactic acid in the urines of rabbits receiving inosite but, since rabbits readily

[1] Anderson, R. J., *J. Biol. Chem.*, 1916, xxv, 391.

[2] Külz, E., Sitzungsber. Ges. Beförd. ges. Naturwiss., Marburg, 1876, No. 4, given in *Maly's Jahresber. Thierchem.*, 1876, vi, 45.

[3] Mayer, P., *Biochem. Z.*, 1907, ii, 393; 1908, ix, 533.

excrete lactic acid under a variety of conditions, this could not be regarded as a strong indication of a relation between inosite and carbohydrates.

In our own experiments we used phlorhizinized dogs. In these animals, although there is a normal amount of glucose in the blood, it apparently cannot be utilized. Moreover, the organism seems to form glucose from every available source. The ratio of glucose to nitrogen in the urine of fasting phlorhizinized dogs remains for days between 3.0 and 3.6 (the variation is much smaller in any one experiment) a value scarcely equalled and, in all probability, never exceeded even in the severest forms of diabetes mellitus. This ratio is assumed to represent the maximum formation of glucose from protein. If, therefore, this ratio rises after the administration of any substance, that substance may be regarded as having been converted into glucose in the organism. There are certain exceptions, such as narcotics and other toxic substances, which liberate the small amounts of glycogen which such phlorhizinized dogs still retain. In such cases the amount of glucose excreted in excess of the usual amount ("extra glucose") bears no constant relation to the amount of material administered and the effect is obtained only with the first few doses and is absent thereafter, for all the available glycogen has been excreted as glucose.[4]

At first the usual technique was employed. After the ratio of glucose to nitrogen in the urine (G:N ratio) had reached a constant level, the inosite, in aqueous solution, was injected subcutaneously. The G:N ratio rose very little. However, it was noticed that it did rise, even though very slightly, in every experiment. Unchanged inosite was also found in the urine (Tables IX and X). It was then determined to test the capacity of the normal dog to oxidize inosite. It was found that about one-half of the inosite administered was excreted unchanged (Table I). It was believed that this poor utilization might be due to the fact that the inosite was rapidly absorbed from the aqueous solution and as rapidly excreted, before the tissues had much opportunity to change it. In order to secure a more gradual absorption of the inosite, it was then given in

[4] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.

suspension in cottonseed oil at intervals of 4 to 6 hours. Determinations of the ratio of total carbon to nitrogen in the urine indicated that a fairly regular excretion of inosite could be secured in this manner (Table II). An experiment with glucose on a phlorhizinized dog showed that a fairly uniform excretion of the added glucose could be secured. Also, the length of the experimental period was increased from 12 hours to at least 18 hours, in which period the inosite was administered at intervals of 4 to 6 hours.

In the normal fasting dog it was found that a considerable portion of the inosite administered could not be accounted for in the urine, either as such or by calculation from the ratio of carbon to nitrogen. Excretion into the intestine was improbable for there were usually no feces. In one experiment (Table VII) the dog was fed a constant mixture of hashed boiled beef, cracker meal, Crisco (a hydrogenated fat), bone ash, and water. The periods were marked off with carmine. Determinations of the total carbon and nitrogen in the feces were made and an attempt was made to isolate inosite from the feces of the experimental period. This was unsuccessful and the ratios of carbon to nitrogen in the feces gave no reason for believing that inosite, or any derivative thereof, was present in the feces.

A simple retention was also unlikely for in only two experiments (Tables IV and VI) was inosite found in the urine more than 12 hours after the close of the experimental period. There was a discrepancy between the amount of inosite calculated from the "extra carbon" and that actually isolated that was somewhat greater than was to be expected from the results obtained in the recovery of inosite added to urine. Among the possible derivatives of inosite were phenols and oxalic and lactic acids. The excretion of none of these was found to be increased (Tables I to IV).[5] It was thought possible that some of the inosite might be excreted, with or without previous change, in combination with sulfuric or glycuronic acids. No increase in the excretion of combined or ethereal sulfuric acid was observed.

[5] Dakin, H. (*J. Biol. Chem.*, 1916-17, xxviii, 429), has also found that the excretion of phenols in dogs is unaffected by the administration of inosite. As regards the excretion of inosite, his results are in accord with those of Anderson.[1]

There was, however, a slight, though unmistakable, increase in the excretion of glycuronic acid (Tables VI and VII). This was determined by the method of Tollens,[6] in which the glycuronic acids are first precipitated with basic lead acetate and ammonium hydroxide, the precipitate is filtered off and washed, and then treated with boiling 12 per cent hydrochloric acid, distilling off the furfurol formed. This is then precipitated as the phloroglucide.[7] There is very little evidence that the substance responsible for this increase is really glycuronic acid. The results are reported in terms of this only as a matter of convenience. That the increase was not due to the presence of inosite or of a reducing substance in the urine or to the administration of cottonseed oil was shown by suitable control experiments (Table VIII). It is very likely that the substance, whatever it may be, is identical with the dextrorotatory non-reducing substance observed by Mayer in the urine of some of the rabbits to which he administered inosite.

In the experiments with phlorhizinized dogs (Tables XI to XV) it was found that the administration of inosite in cottonseed oil was followed by a slight though unmistakable increase in the glucose: nitrogen ratio. Inosite is not a toxic or narcotic substance and the "extra glucose" can hardly be regarded as being derived from the glycogen or other carbohydrate of the body. Moreover, the amount of "extra glucose" increased as the experiment was continued, leaving very little doubt that the glucose was actually derived from the inosite administered. That it was not due to the cottonseed oil was shown by two experiments. In one of these (Table XIV) the dog received injections of cottonseed oil alone for four 12 hour periods and then, after a 24 hour interval, the inosite was administered in the usual manner. The oil alone produced only the slightest, if any, rise in the glucose: nitrogen ratio. In the inosite period, however, the usual rise was observed. In the other control experiment (Table XV) the dog received 15 cc. of cottonseed oil every 6 hours during the fore- and after-periods. This was at least

[6] Tollens, C., *Z. physiol. Chem.*, 1909, lxi, 95.

[7] In these experiments the paper was not included in the mixture subjected to distillation, thus avoiding the large correction employed by Tollens.

50 per cent more oil than was used for the suspension of the inosite. Nevertheless the glucose: nitrogen ratio rose in the usual manner with the administration of the inosite and fell [illegible].

The excretion of acetone and of β-hydroxybutyric acid was [illegible] diminished in the experimental periods.

Determinations were made of the total carbon in the urine. From this amount were deducted the amounts of carbon present as glucose, as acetone, and as β-hydroxybutyric acid. From the residual value ("rest C"), a carbon: nitrogen ratio was calculated. From this, following the method introduced by Lusk for the calculation of the "extra glucose," the "extra carbon" and the inosite equivalent thereto were calculated. The amount of inosite actually found corresponded, as well as could be expected, with the amount calculated in this manner. In only one experiment (Table XIV) was this not true. In this experiment, particularly in one period, the amount of "extra carbon" was very high. It is possible that the excretion of some unknown constituent was very irregular, giving rise to the results obtained.

If the amount of "extra glucose" be added to the amount of inosite calculated from the "extra carbon," the sum, except in the experiment already alluded to, is almost exactly equal to the amount of inosite administered. In view of the error inherent in these determinations and calculations, the correspondence is surprisingly close. Apparently, therefore, inosite, though slowly and incompletely, is converted molecule for molecule into glucose.

EXPERIMENTAL.

The general plan of the experiments and the analytical methods were those generally employed in this laboratory.[8]

Total carbon was determined by oxidation with sulfuric acid and potassium dichromate, passing the gaseous products of oxidation through a heated combustion tube containing copper oxide and lead chromate, drying with calcium chloride, and finally absorbing the carbon dioxide in soda-lime.

Inosite was prepared from "steep water" by the method of

[8] Greenwald, I., *J. Biol. Chem.*, 1914, xviii, 115; 1916, xxv, 81.

Griffin and Nelson.[9] The isolation from the urine was accomplished by Mayer's method, crystallizing the inosite from alcohol and from acetic acid. In the experiments with phlorhizinized dogs this crystallization was repeated several times before the product was considered pure enough to weigh. The method of Meillère and Fleury[10] gave a purer product but the yield was much lower and extremely variable.

Oxalic acid was determined by Dakin's method[11] and phenols by the method of Folin and Denis.[12]

TABLE I.

Excretion of Inosite after Administration in Aqueous Solution. Normal Fasting Dogs. 12 Hour Periods.

Nitrogen.	Carbon.	C:N.	"Extra carbon."	Inosite.	Oxalic acid.	Remarks.
gm.	gm.		gm.	gm.	gm.	
0.852	0.589	0.692				
0.929	2.114	2.276	1.475	3.71		Weight 4.15 kilos. 5.81 gm. inosite in 100 cc. H_2O in 6 hourly doses in first half of period.
0.901	0.648	0.719	0.028	0.07		
0.962	0.657	0.683				
0.803	0.700	0.873			0.008	
0.996	0.859	0.864			0.020	
1.100	2.950	2.683	2.00	5.06	0.010	Weight 5 kilos. 8.05 gm. inosite in 100 cc. H_2O in 5 hourly doses in first half of period.
1.156	0.976	0.844			0.010	

The column headed inosite gives the amount of inosite calculated from the "extra carbon." The isolation of inosite from the urine was unsatisfactory because of mechanical losses, etc.

[9] Griffin, E. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1915, xxxvii, 1552. We are indebted to the Corn Products Refining Company for the supply of steep water.

[10] Meillère, G., and Fleury, P., *J. pharm. et chim.*, 1910, series 7, i, 348.

[11] Dakin, H. D., *J. Biol. Chem.*, 1907, iii, 77.

[12] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.

TABLE II.

Hourly Excretion of Inosite after Administration in Suspension in Cottonseed Oil. Dog 11.

Length of period.	Nitrogen.	Carbon.	C : N.	"Extra carbon."	Inosite.	Oxalic acid.	Remarks.
hrs.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	
12	0.963	0.873	0.906			0.011	
12	0.826	0.728	0.882				
2	0.175	0.259	1.49	0.102	0.256		Weight 8 kilos. 4 gm. inosite in 10 cc. cottonseed oil.
2	0.183	0.376	2.06	0.212	0.533		
2	0.190	0.658	3.46	0.487	1.223		4 gm. inosite in 10 cc. cottonseed oil.
2	0.192	0.791	4.03	0.600	1.508		
2	0.175	0.547	3.14	0.390	0.981		
2	0.144	0.316	2.19	0.186	0.468		
2	0.169	0.261	1.54	0.108	0.272		
Total.....	1.208	3.208		2.085	5.241		
Composite...	1.228	3.267		2.172	5.461	0.005	
10	1.180	1.201	1.017	0.138	0.347	0.008	
12	1.347	1.207	0.896			0.009	

A composite of the 2 hour urines was prepared and analyzed, with the results given above. The values for inosite are those calculated from the amount of "extra carbon." 4.4 gm. of inosite were isolated from the composite urine.

TABLE III.

Excretion of Inosite after Administration in Oil for Four 12 Hour Periods. Dog 17, Normal, Fasting, Weight 9 Kilos.

Nitrogen.	Carbon.	C : N.	"Extra carbon."	Oxalic acid.	Inosite.		
					Calculated.	Found.	Administered.
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
2.096	1.571	0.750		0.0045			
1.591	1.371	0.861		0.0030			
1.415	1.579	1.116	0.306	0.0023	0.769	0.50	4.85
1.639	2.874	1.754	1.400	0.0034	3.520	2.70	6.00
1.930	2.944	1.519	1.200	0.0020	3.017	3.05	6.00
2.470	3.710	1.514	1.505	0.0017	3.780	3.10	6.00
2.305	2.210	1.000	0.221	0.0045	0.554	0.34	
1.575	1.550	0.987	0.137	0.0023	0.350	0.00	
1.621	1.506	0.929					
1.300	1.230	0.940					
Total.....					11.99	9.69	22.85

TABLE IV.

Excretion of Inosite after Administration in Oil for Four 12 Hour Periods. Dog 21, Normal, Fasting, Weight 12 Kilos.

Nitrogen.	Carbon.	C : N.	"Extra carbon."	Phenols.	Inosite.		
					Calculated.	Found.	Administered.
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.156	0.981	0.848		0.045			
1.034	0.965	0.933		0.049			
0.995	2.289	2.301	1.39	0.047	3.50	2.56	9.2
0.932	3.600	3.864	2.76	0.058	6.94	5.33	8.0
1.448	3.926	2.711	2.62	0.066	6.59	5.40	9.0
1.422	4.023	2.829	2.74	0.066	6.90	5.10	8.0
1.311	1.802	1.375	0.62	0.054	1.56	1.30	
1.259	1.225	0.973	0.09	0.057	0.23	0.35	
1.280	1.354	1.058	?*	0.068	?*	0.13	
1.144	1.023	0.895		0.067			
Total.....					25.72	20.17	34.2

*The urine was contaminated with blood from a wound. The C : N ratio is probably too high and the "extra carbon" and inosite have therefore not been calculated.

TABLE V.

Excretion of Inosite after Administration in Oil for Four 12 Hour Periods. Dog 25, Normal, Fasting, Weight 20 Kilos.

Nitrogen.	Carbon.	C : N	"Extra carbon."	Inosite.		
				Calculated.	Found.	Administered.
gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*
2.360	2.113	0.895				
2.565	2.240	0.873				
2.888	3.100	1.073	0.965	2.43	1.70	8.46
3.637	3.947	1.085	1.258	3.16	2.50	8.00
5.057	5.103	1.002	1.330	3.34	3.37	8.00
5.642	7.062	1.252	2.894	7.28	6.77	8.00
4.970	3.802	0.765				
4.431	3.103	0.700				
2.528*	1.902	0.752				
Total.....				16.21	14.34	32.46

*10 hour period.

The "extra carbon" and inosite have been calculated upon the basis of a basal C : N ratio of 0.739, the average of the last three periods. This gives the maximal values for "extra carbon" and inosite.

TABLE VI.

Excretion of Inosite after Administration in Oil for Four Successive 12 Hour Periods. Dog 25, Weight 19 Kilos.

Nitrogen.	Carbon	C : N.	"Extra carbon."	Inosite.			Sulfur.		Glycuronic lactone	Period.
				Calculated.	Found.	Administered.	Inorganic.	Combined SO_4.		
gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	hrs.
1.052	1.502	0.732					0.084	0.016	0.125	10.5
1.536	1.781	0.763					0.097	0.018	0.148	12
3.622	3.676	1.402	1.72	4.32	3.84	12.4	0.091	0.017	0.189	12
3.624	4.528	1.727	2.57	6.46	5.99	10.0	0.092	0.016	0.176	12
3.926	5.829	1.487	3.29	8.28	6.94	12.0	0.131	0.016	0.250	12
4.075	6.451	1.583	3.81	9.58	7.67	10.0	0.118	0.012	0.232	12
1.550	3.857	0.848	0.91	2.29	1.74		0.164	0.015	0.211	12
1.774	3.401	0.712	0.31	0.77	0.31		0.163	0.014	0.162	13
3.474	2.248	0.647							0.165	10
4.301	2.789	0.649								14.5
Total..	.....	.....	12.61	31.70	26.49	44.4				

The basal C : N ratio has been taken as 0.747 (the average of the ratios in the fore-periods) for the first two experimental periods and as 0.648 (the average of the ratios in the last two periods) for the others.

TABLE VII.

Excretion of Inosite after Administration in Oil for Four 24 Hour Periods. Dog 25, Weight 19 Kilos. Fed Daily..

Food:

	gm.
Boiled beef	190
Cracker meal	76
Crisco	57
Bone ash	19
Water	760 cc.

Nitrogen.	Carbon.	C : N.	"Extra carbon"	Inosite.			Sulfur.		Glycuronic lactone.	Feces.		
				Calculated.	Found.	Administered.	Inorganic.	Combined SO_4.		Carbon.	Nitrogen.	C : N.
gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
11.10	7.18	0.647					0.452	0.076	0.338			
9.75	6.30	0.646					0.358	0.078	0.320	20.7	1.61	12.9
9.22	9.92	1.076	3.93	9.87	8.32	20.9	0.323	0.061	0.489			
9.87	12.23	1.239	5.81	14.61	13.89	22.0	0.286	0.051	0.464			
10.51	12.68	1.207	5.85	14.72	13.87	22.0	0.379	0.060	0.412			
11.87	11.79	0.993	4.07	10.24	8.01	22.0	0.443	0.045	0.522	34.4	3.38	10.2
10.49	6.87	0.655					0.412	0.049	0.280			
9.47	6.82	0.652					0.441	0.049	0.309	16.6	1.73	9.6
Total	.....	.....	.....	49.44	44.09	86.9						

TABLE VIII.

Effect of Subcutaneous Administration of Cottonseed Oil on the Excretion of Glycuronic Acid. Dog 25, Weight 19 Kilos. Fed Daily. Periods 24 Hours Each.

Food:

	gm.
Boiled beef	190
Cracker meal	76
Crisco	57
Bone ash	19
Water	760 cc.

Nitrogen.	Glycuronic lactone.	Cottonseed oil.
gm.	gm.	cc.
11.56	0.300	
10.97	0.348	
10.25	0.325	4 × 20
10.57	0.349	4 × 20
9.90	0.308	3 × 20
10.31	0.349	4 × 20
Lost.		
9.97	0.355	
9.01	0.308	

TABLE IX.

Inosite Administered in Aqueous Solution to a Phlorhizinized Dog, Weight 9.5 Kilos. Periods 12 Hours Each.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Doses administered.
gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
10.08	32.5	30.4	3.22		0.091	0.441	19.39	6.21	0.617				
8.95	29.1	28.1	3.25		0.091	0.573	17.02	5.10	0.572				
6.92	24.9	24.8	3.61	1.94	0.065	0.482	19.64	9.46	1.365	5.04	12.7	8.6	14.4
7.18	24.2	23.6	3.37		0.077	0.448	14.76	4.87	0.678				
5.83	20.4		3.49		0.094	0.629	12.44	4.00	0.686				

TABLE X.

Inosite Administered in Aqueous Solution to a Phlorhizinized Dog, Weight 12 Kilos. Periods 12 Hours Each.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.
5.183*	18.57	18.6	3.39		0.039	0.120	11.15	3.68	0.671				
6.365	19.60	19.6	3.16		0.060	0.231	12.52	4.58	0.738				
5.915	22.36	22.8	3.78	2.49	0.049	0.215	18.01	8.98	1.519	4.62	11.6	8	12.7
5.817	19.94	19.9	3.43		0.139	0.793	12.67	4.29	0.738				
6.355	22.01	21.8	3.46		0.099	0.430	13.65	4.64	0.730				

*11 hour period.

TABLE XI.

Inosite Administered in Suspension in Oil to a Phlorhizinized Dog, Weight 20.6 Kilos. Periods 12 Hours Each.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.
4.635*	16.34	15.9	3.53		0.197	2.37	12.09	4.37	0.943				
7.026	25.62	24.5	3.65		0.173	3.08	17.63	5.97	0.843				
6.852	27.10	26.3	3.95	2.12	0.236	2.27	19.75	7.78	1.136	2.34	5.88	5.12	10.5
7.183	26.09	25.4	3.63		0.319	3.14	18.13	6.10	0.849	0.39	0.98	0.58	
7.150	25.66		3.59		0.194	2.72	17.10	5.53	0.791				
4.576**	14.69		3.21		0.084	0.62	9.83	3.65	0.798				
Total...	.....	.....	.....	.....	.....	.....	.....	.....	.....	2.73	6.86	5.70	

*8 hour period.

**6 hour period.

The "rest" carbon and the inosite have been calculated upon the basis of a basal C : N ratio of 0.795, which gives the maximal values for "extra" carbon and inosite.

TABLE XII.

Inosite Administered in Suspension in Oil to a Phlorhizinized Dog, for Four Successive 12 Hour Periods.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.
7.101	24.57	24.7	3.46										
7.068	26.06	26.4	3.68		0.451	4.26	16.74	4.15	0.587				
7.244	24.65	24.7	3.40		0.525	4.57	17.92	5.68	0.784				
7.278	28.70	29.1	3.77	0.51	0.181	4.72	22.24	8.53	1.172	2.71	6.81	4.15	10.45
6.937	27.62	28.6	3.98	1.94	0.169	2.66	20.22	7.90	1.139	2.35	5.88	3.81	10.45
6.352	25.39	21.2	4.00	3.68	0.079	0.82	18.37	7.84	1.234	2.76	6.93	4.54	12.45
5.820	24.92	24.7	4.28	3.84	0.060	0.51	18.52	8.23	1.414	3.57	8.99	6.76	10.45
5.205	22.18	21.9	4.36	0.85	0.013	0.07	14.75	5.89	1.132	1.73	4.34	2.10	
3.854	14.89	14.9	3.87		0.007	0.03	9.22	3.18	0.825				
3.226	11.77	11.2	3.74				6.98	2.28	0.707				
1.651	6.18		3.75										
Total..	.....	.....	.....	10.82						13.12	32.95	21.36	43.80

The calculations have been made upon the basis of basal ratios of 3.70 and 0.800 for G : N and C : N, respectively. It is believed that these ratios give minimal values for "extra glucose" and maximal values for inosite.

TABLE XIII.

Inosite Administered in Suspension in Oil to a Phlorhizinized Dog, Weight 15 Kilos, for Four Successive 12 Hour Periods.

Nitrogen.	Glucose.		G : N.	"Extra glucose."	Acetone.	β-Hydroxybutyric acid.	Carbon.				Inosite.		
	Benedict.	Polariscope.					Total.	"Rest."	C : N.	"Extra."	Calculated.	Found.	Administered.
gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.
3.851*	12.81	12.3	3.33		0.044	0.167	7.11	1.91	0.495				
7.658	24.40	23.3	3.19		0.120	0.463	14.01	4.02	0.525				
8.395	27.54	27.3	3.28	0.92	0.128	0.829	19.13	7.72	0.920	1.01	2.53	2.62	10.2
8.160	28.52	27.2	3.49	2.61	0.133	0.544	21.11	9.44	1.157	2.10	5.27	6.63	12.4
7.788	27.46	26.5	3.53	2.80	0.087	0.339	21.31	10.18	1.307	3.95	9.93	7.55	12.1
7.359	25.54	26.3	3.47	2.21	0.102	0.308	18.96	8.60	1.169	2.72	6.83	6.53	9.0
7.349	25.23	24.9	3.43	1.91	0.092	0.293	17.04	6.66	0.906	0.78	1.96	2.18	
7.148	22.61	22.7	3.16		0.097	0.292	15.43	6.24	0.873	0.52	1.31	0.00	
6.315	19.95	20.5	3.16		0.051	0.222	12.94	4.87	0.772				
5.708	19.03		3.33		0.054	0.156	12.56	4.89	0.857				
Total..	.....	.....	.....	10.45						11.08	27.83	25.51	43.7

*6 hour period.

The "extra glucose" has been calculated upon the basis of a dominant ratio of 3.17. The wide variations in the value of the C : N ratio made the selection of a dominant ratio difficult. The value finally selected and used was 0.80.

TABLE XIV.

Effect of Administration of Cottonseed Oil Alone, and of Inosite in Oil, to a Phlorhizinized Dog, Weight 11.6 Kilos. Periods 12 Hours Each.

	Glucose.				Carbon.				Cottonseed oil.
	Polarized.	Polariscope.	G : N.	"Extra glucose."	Total.	"Rest."	C : N.	"Extra."	
	gm.	*gm.*		*gm.*	*gm.*	*gm.*		*gm.*	*cc.*
9.559	32.36		3.39						
4.812*	16.74		3.48						
4.931*	17.29		3.51						
10.02	36.31		3.62						40
9.561	35.04		3.67						60
9.504	33.48		3.52						40
8.857	30.19		3.41						60

									Inosite.		
									Calculated.	Found	Administered.
8.478	29.72		3.51								
3.950*	13.44	13.0	3.40		8.68	3.34	0.844		*gm.*	*gm.*	*gm.*
4.197*	14.22	14.5	3.30		8.92	3.26	0.778				
7.774	30.30	29.1	3.90	6.10	20.17	8.12	1.045	2.00	5.02	3.18	7.88
7.723	28.65	28.7	3.71	4.09	20.49	9.10	1.178	3.01	7.57	3.36	12.0
6.656	26.85	26.4	4.04	5.72	18.36	7.68	1.154	2.43	6.12	3.12	10.0
5.793	21.98	21.1	3.80	3.59	16.92	8.18	1.412	3.62	9.09(?)**	2.83	12.0
5.124	18.63	17.6	3.64	2.35	12.72	5.31	1.061	1.39	3.52	0.18	
2.743*	8.42		3.07		5.51	2.16	0.784				
3.128*	9.21		2.94		5.99	2.33	0.745				
5.916	17.33		2.91								
Total..	.....	.....	.....	21.85				12.45	31.32	12.67	41.88

*6 hour period.

**Note the unusual discrepancy between amount of inosite calculated from "extra" carbon and that actually found.

TABLE XV.

[illegible] Suspended in Cottonseed Oil, Administered to a Phlorhizinized Dog [illegible] Oil [illegible] the Experiment. Dog 29, Weight [illegible] Kilos.

[illegible]	Glucose.		[illegible]	[illegible]	Carbon.				Inosite.		
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	"[illegible]"	[illegible]	"Extra"	Calculated.	Found.	Administered.
[illegible]	[illegible]	*gm.*		[illegible]	*gm.*	*gm.*		[illegible]	*gm.*	[illegible]	*gm.*
[illegible]	19.[illegible]		3.[illegible]								
[illegible]	[illegible]	19.[illegible]	3.14								
12.[illegible]	[illegible].86	35.[illegible]	[illegible].97		23.[illegible]	[illegible]	[illegible]				
11.[illegible]	[illegible]	36.7	3.[illegible]	[illegible].61	[illegible]	[illegible]	[illegible]	1.10	2.7[illegible]	1.[illegible]	[illegible]
11.[illegible]	[illegible]	37.9	[illegible]	[illegible]	[illegible]	11.[illegible]	1.662	[illegible]	7.17	6.43	10.0
10.[illegible]	[illegible]	53.1	3.56	[illegible].07	26.[illegible]	[illegible]	1.0[illegible]	2.0[illegible]	7.[illegible]	6.[illegible]	[illegible]
10.[illegible]	[illegible]	37.[illegible]	[illegible]	[illegible]	[illegible].75	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	10.0
9.[illegible]	[illegible].77	33.[illegible]	3.[illegible]	5.95	[illegible].75	[illegible]	[illegible]				
[illegible]	20.65	27.8	3.[illegible]		[illegible]	[illegible]	[illegible]				
Total.	......	......	......	[illegible]				[illegible]	[illegible]	[illegible]	[illegible].7

J. Biol. Chem. 162:77-85,1946

DIETARY FACTORS IN THE REGULATION OF LIVER LIPID CONCENTRATION

By PHILIP HANDLER

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, October 20, 1945)

The administration of a liver extract (1) or biotin (2) to rats which had previously been fed a low protein diet with no B vitamin supplement for 3 weeks has been observed to result in fatty livers despite the presence of an adequate supply of dietary choline. However, when inositol was also included in the diet, normal liver fat concentrations were found (3). The present paper describes an attempt to determine whether biotin or any other factors present in the liver extract play specific rôles in the regulation of the liver lipids or whether they simply increase the demand for lipotropic factors by stimulating the appetite and growth of previously stunted rats. It was also hoped that the data might explain the need for dietary inositol under such conditions, since choline and methionine suffice to provide normal fat concentrations in the livers of rats fed the usual synthetic rations, and to indicate whether other dietary factors such as tocopherol, vitamin K, and the unsaturated fatty acids also function in this system.

EXPERIMENTAL

The experimental procedure followed, as closely as possible, that used by McHenry and his coworkers (1–5). Male rats of the Vanderbilt strain (6) were grown to a weight of 80 gm. and then housed in individual cages and placed upon the depletion diet (A) for 3 weeks. Less than 6 per cent of the animals failed to survive this period, while the survivors usually weighed between 55 and 70 gm. They were then offered the diets shown in Table I and in addition all animals were given the following supplement by subcutaneous injection daily: thiamine 25 γ, riboflavin 30 γ, calcium pantothenate 100 γ, pyridoxine hydrochloride 20 γ, nicotinamide 50 γ, *p*-aminobenzoic acid 100 γ. The doses of the other supplements, when given, were choline 30 mg., inositol 30 mg., biotin 5 γ, folic acid 5 γ, α-tocopherol 200 γ, and naphthoquinone acetate 20 γ; and 0.1 cc. of refined corn oil was used as a source of unsaturated fatty acids. Both crystalline folic acid and a potent liver concentrate were used but no differences were observed in their effects. The liver extract was prepared by the procedure of McHenry and Gavin (1) and, when desired, 0.5 cc. was mixed with about 2 gm. of the proper diet and placed on top of the remainder of the

food in each individual feeding cup. This amount of extract provided about 5.5 mg. of choline, less than 2 mg. of inositol, about 2 γ of biotin, and appreciable quantities of folic acid. The latter was assayed against another liver standard and it is not possible to state the actual quantity present. All animals were sacrificed after 7 days, except when stated otherwise, and liver samples taken for analysis. Fatty acids were determined in the usual fashion (8) and cholesterol was estimated by the procedure of Schoenheimer and Sperry (9). The results are summarized in Tables II and III. Each value is the mean for a group of ten rats.

When the low fat diet (B) was fed with only the basal supplement, fatty livers were obtained which were prevented by choline. Inositol appeared to have some lipotropic activity but it was not as marked as that of choline. The administration of liver extract resulted in fatty livers which were somewhat diminished in fat content by additional choline (the extract provided only 5 mg. of choline per day) and more so by inositol, but prevented only by a combination of the two. When biotin was given in addition to the basal supplement, there were again found fatty livers partially resistant to the action of either choline or inositol alone but susceptible to their combined action. However, the biotin effect was not as pronounced as that of the liver extract in stimulating appetite, growth, or liver lipid content. These findings are all essentially in agreement with the findings of McHenry and Gavin (1–4). Since it seemed possible that folic acid might be the substance in liver extract which accounted for the difference between its action and that of biotin, the effects of this compound were studied. When given with the basal supplement, however, it appeared to have no effect on any of the factors which were measured and so it was tried in combination with biotin. The results with this combination were almost identical with those observed when the liver extract was used.

When the high fat diet (Diet C) was employed, it was found that even

TABLE I

Composition of Diets

	Diet A	Diet B	Diet C	Diet D	Diet E
Casein	10	10	10	10	12
Sucrose	84	83.6	63.1	84	71.1
Crisco			20		10
Salts (Hubbell *et al.* (7))	4	4	4	4	4
Cellulose	2	2	2	2	2
Sulfasuxidine				1	1
Cystine		0.4	0.4		0.4
Cholesterol			0.5		0.5

without liver extract, biotin, or folic acid, the presence of both choline and inositol was required for the maintenance of normal liver lipid concentrations. It should be noted that Diet C provided 4.5 kilocalories per gm., while Diet B provided 3.7 kilocalories per gm. The effects of the liver extract and of the biotin-folic acid combination were even more pronounced on the high than the low fat diet; so that the administration of choline plus inositol did not provide normal liver lipid concentrations until the 2nd week. Of considerable interest was the observation that on both diets supplementary choline alone was sufficient to obtain normal lipid concentrations even in the presence of liver extract or biotin plus folic acid if the experiment was continued for 24 days. It should also be noted that no differential behavior between cholesterol and liver fat was found. The livers of highest fat content contained the greatest amount of cholesterol and livers of normal fatty acid concentration also were of normal cholesterol content.

TABLE II

Lipotropic Activity of Choline and Inositol on Low Fat Diet B Plus Basal Vitamin Supplement

Group No.	Supplement	Weight change	Food intake	Liver fatty acids, mean and standard error	Liver cholesterol
		gm.	*gm. per day*	*per cent wet weight*	*per cent wet weight*
1	None	9.1	6.0	18.7 ± 3.1	0.51
2	Choline	11.7	5.9	5.3 ± 0.4	0.42
3	Inositol	11.2	5.7	16.8 ± 2.9	0.39
4	Liver	22.3	11.6	23.4 ± 3.6	0.93
5	" + choline	24.4	11.1	20.7 ± 1.9	0.82
6	" + inositol	19.7	12.2	8.4 ± 0.6	0.47
7	" + " + choline	23.6	11.9	4.8 ± 0.3	0.36
8	Folic acid	10.3	6.1	20.2 ± 1.7	0.56
9	Biotin	16.8	7.8	19.6 ± 2.4	0.74
10	" + choline	16.0	7.9	8.8 ± 0.5	0.69
11	" + inositol	17.2	7.7	13.6 ± 1.8	0.55
12	" + " + choline	17.6	8.3	4.6 ± 0.3	0.32
13	" + folic acid	21.7	10.8	21.7 ± 3.5	0.88
14	" + " " + choline	19.9	11.7	18.4 ± 2.7	0.80
15	" + " " + inositol	19.0	12.9	17.8 ± 1.9	0.49
16	" + " " + " + choline	21.1	11.9	5.3 ± 0.3	0.31
17	Biotin + folic acid + inositol + tocopherol	19.6	11.8	10.4 ± 0.7	0.41
18	Biotin + folic acid + inositol + corn oil	18.8	12.1	22.0 ± 1.5	0.66
19	Biotin + folic acid + choline 24 days	57.4	8.7	6.1 ± 0.4	0.35

Since biotin, folic acid, and inositol are normally available to the rat from synthesis by the intestinal microorganisms, it seemed possible that the effects of these substances might better be studied in animals in which the intestinal synthesis was inhibited by the incorporation of dietary sulfasuxidine (succinylsulfathiazole). The technique was similar to that above. All animals were depleted for 3 weeks on Diet D which differed from Diet A only in that it contained sulfasuxidine, and then were switched to Diet E which also contained sulfasuxidine and whose fat content was intermediary

TABLE III

Lipotropic Activity of Choline and Inositol on High Fat Diet C Plus Basal Vitamin Supplement

Group No.	Supplements	Weight change	Food intake	Liver fatty acids, mean and standard error	Liver cholesterol
		gm.	*gm. per day*	*per cent wet weight*	*per cent wet weight*
1	None	22.1	6.3	26.1 ± 3.6	0.94
2	Choline	23.3	5.9	11.8 ± 0.8	0.76
3	Inositol	20.8	6.0	17.2 ± 1.9	0.69
4	" + choline	23.8	6.4	7.3 ± 0.6	0.39
5	Liver	40.8	10.4	27.3 ± 4.4	1.37
6	" + choline	36.4	11.1	19.9 ± 3.1	0.90
7	" + inositol	39.1	10.7	14.9 ± 1.7	0.74
8	" + " + choline	42.2	11.4	11.7 ± 0.8	0.63
9	" + " + " 14 days	73.0	9.6	5.8 ± 0.3	0.37
10	Biotin + folic acid	37.9	11.2	25.9 ± 3.7	1.22
11	" + " " + choline	38.4	10.8	18.1 ± 1.6	0.90
12	" + " " + " 14 days	66.8	9.6	11.2 ± 0.6	0.69
13	" + " " + " 24 "	79.3	8.9	6.0 ± 0.5	0.34
14	" + " " + inositol	35.1	10.4	19.7 ± 2.4	0.77
15	" + " " + " + choline	40.2	11.3	10.3 ± 1.8	0.54
16	Biotin + folic acid + inositol + choline 14 days	64.9	10.0	5.1 ± 0.3	0.33

between that of Diets B and C but did contain cystine and cholesterol. The same basal supplement was also given to these animals but without *p*-aminobenzoic acid. The results are summarized in Table IV. Each figure represents the mean of a group of ten rats.

The results were essentially in agreement with those found previously. Again choline alone was sufficient to maintain normal liver lipid concentrations (Group 2) unless biotin and folic acid were present (Groups 8 and 15). The effects of biotin alone (Group 10), however, were less notable than in the previous experiments, presumably because of the diminished supply of

folic acid from intraintestinal synthesis, while folic acid alone (Group 11) exerted even less effect than biotin alone. The combination of choline plus inositol always served to regulate the liver lipids unless unsaturated fatty acids were given (Group 9) or tocopherol were lacking (Group 13). However, neither of these effects was very marked and may not be real. No effect ascribable to the presence or absence of vitamin K was noted, although it must be stated that no prothrombin time determinations were performed on the blood of animals in Group 16, and in fact

TABLE IV

Effect of Sulfasuxidine on Liver Fat Concentration

All animals received Diet E + the basal vitamin supplement.

Group No.	Choline	Inositol	Biotin	Folic acid	Tocopherol	Naphthoquinone	Corn oil	Food intake	Weight change	Liver fatty acids, mean and standard error	No. of rats with hemorrhagic kidneys
								gm. per day	*gm.*	*per cent*	
1	0	0	0	0	0	0	0	5.8	15.2	18.2 ± 2.2	2
2	+	0	0	0	0	0	0	5.9	14.0	4.8 ± 0.3	0
3	0	+	0	0	0	0	0	5.6	15.7	10.1 ± 1.1	2
4	0	+	0	0	0	0	+	5.2	13.7	22.2 ± 3.4	4
5	0	0	+	+	+	+	0	8.8	26.1	27.4 ± 3.2	6
6	+	+	+	+	+	+	0	10.2	37.6	7.1 ± 0.6	0
7	0	+	+	+	+	+	0	9.9	32.4	23.2 ± 2.7	4
8	+	0	+	+	+	+	0	9.6	29.1	16.4 ± 1.8	1
9	+	+	+	+	+	+	+	10.6	35.3	8.2 ± 0.8	0
10	+	0	+	0	+	+	0	7.3	21.5	8.1 ± 1.0	0
11	+	0	0	+	+	+	0	7.0	23.7	7.0 ± 0.3	0
12	+	0	0	0	+	+	0	5.4	14.6	5.0 ± 0.2	0
13	+	+	+	+	0	+	0	10.0	28.1	9.2 ± 0.5	2
14	0	+	+	+	0	+	0	9.3	26.4	28.5 ± 3.6	7
15	+	0	+	+	0	+	0	9.9	30.1	21.0 ± 3.1	4
16	+	+	+	+	+	0	0	10.4	34.0	7.1 ± 0.4	0
17	+	0	+	+	+	+	0	7.9	71.3	8.9 ± 0.7	0 (24 days)

no specific deficiency symptoms of biotin, folic acid, or inositol ever were noted. In contrast to the former results, however, was the finding that rats given choline, folic acid, and biotin still appeared to require dietary inositol after 24 days (Group 17).

The inhibition of the lipotropic action of inositol by unsaturated fatty acids reported by Beveridge and Lucas (10) was confirmed in both series of experiments (Table II, Group 18; Table IV, Group 4). Some suggestive evidence of a synergism between inositol and tocopherol was also obtained in both series. This can be seen in Table II by comparing Groups 15

and 17. In the sulfasuxidine series, when biotin and folic acid were given, in the absence of choline, inositol plus tocopherol (Group 7) was slightly more effective in regulating the liver lipid than was inositol alone (Group 14). Further, even when choline was included with biotin, folic acid, and inositol, slightly fatty livers were found when no tocopherol was given (Group 13), while when choline and tocopherol were not included in the supplement the highest concentrations of liver lipids and the greatest incidence of hemorrhagic kidneys were observed (Group 14). However, there was no evidence that tocopherol exerted any lipotropic influence of itself (Group 5).

DISCUSSION

In the recent work of MacFarland and McHenry (5) it was found that, while inositol alone was sufficient to control liver lipid concentrations when liver extract was administered, the combination of choline and inositol was required when crystalline biotin was given. On this basis the authors sought to distinguish between the biological activity of the liver extract and that of biotin and also between the mechanisms involved in the lipotropic actions of choline and inositol. However, the liver extract used contained about 20 mg. of choline per cc. according to Gavin and McHenry (2) and since the dose used by the former authors was 2.0 cc. per day they supplied approximately 40 mg. of choline per day in the liver extract. In consequence, the addition of further choline to the diet could not have been expected to have any lipotropic action and only inositol was required to secure normal liver lipid concentrations. In our present work, the liver extract provided only 5.5 mg. of choline per day; so that further choline as well as inositol was required to regulate the liver lipids.

While biotin and folic acid, under the specific conditions employed in this work, definitely increased the demand for dietary lipotropic factors, there is no evidence that either of these substances actually plays a specific rôle in lipid metabolism. When liver extract or biotin plus folic acid as well as the basal supplement was included in the diet of rats which had previously existed on a diet seriously deficient in all of these factors, there occurred a much greater increase in food consumption and growth than that seen when only those members of the vitamin B complex which are normally required to be present in the diet of the rat (basal supplement) were given. Under these circumstances then it would seem reasonable to expect an increased demand for lipotropic factors; *i.e.*, choline and inositol. The demand for inositol diminished as the appetite and growth stimulation by the folic acid-biotin combination tapered off. This occurred when the rats had attained a size not much smaller than that of animals of a comparable age on the same basal diet (which is low in protein) supplemented with the basal vitamin mixture but never previously depleted. At this point also it seems likely that the intestinal flora which normally provides the biotin, folic acid, and inositol for rats on diets devoid of these factors is again established and capable of meeting the requirements for these substances. This is borne out by the finding that when sulfasuxidine, folic acid, and biotin were given there was still a small demand for inositol after 24 days. Such a point of view seems reasonable also when one considers the demand for inositol as well as choline when the high fat diet was fed without any liver extract, biotin, or folic acid. It is consistent also with the previous observations (8, 11–13) that fatty livers due to choline deficiency are not found in animals which are, for any reason, losing weight and that the extent of the accumulation of liver fat in choline deficiency is roughly proportional to the food consumption and growth rate, particularly in young rats. From this standpoint, there is no real justification for the use of the term "biotin fatty liver."

Probably the significance of the present study lies in the suggestion that a diet which is seriously inadequate in the usual accessory food factors may not only result in the deterioration of the host animal but also of the symbiotic microorganisms in the bowel and thus produce a deficiency of the factors ordinarily provided by these organisms for their host. Further, since there is an increased appetite and over-all metabolism during the period of convalescence from any disease in which there is appreciable weight loss, there may then occur an increased demand and consequent relative deficiency in the supply of those vitamins provided by the intestinal organisms, as their synthetic powers may well be limited.

The present work does confirm the lipotropic action of inositol, although it does not suggest any mechanism for this behavior. In contrast to previous suggestions (3, 5) the data do indicate that inositol exerts no selective activity for cholesterol esters rather than the glycerides. The possibilities remain that it functions either by the formation and transport of inositol-containing phospholipids or as a phosphorylating catalyst, perhaps similar to phytic acid, which accelerates the formation of the nitrogenous phospholipids. This possibility is now being investigated. However, it does appear that even in the absence of dietary choline inositol exerts lipotropic activity. The antagonistic action of the highly unsaturated fatty acids was also confirmed.

Of interest is the repeated suggestion that tocopherol may also function in this system. The effects noted, while quite small, were consistent and in keeping with evidence from other sources. Thus Milhorat and Bartels (14) have suggested that a tocopherol-inositol ether may be an active agent in reducing the creatinuria of human muscular dystrophy. Dam and Glavind (15) have found that inositol prevents the exudative diathesis of

vitamin E-deficient chicks, a form of capillary damage which may be similar or analogous to the hemorrhagic kidneys of choline-deficient rats, and that inositol prevents the deposition in the soft tissues of vitamin E-deficient animals (16) of a brown pigment somewhat similar to the ceroid in cirrhotic livers due to choline deficiency (17). In both instances the accumulation of this pigment is dependent upon a source of unsaturated acids, usually a fish liver oil (16, 18, 19). The deposition of such a pigment in the muscle, both smooth and striated, of vitamin E-deficient animals has been a consistent finding (20). While Dam has stated that no tocopherol was found in an alkaline hydrolysate of a lipocaic preparation (16), this problem warrants further investigation, since inositol has been implicated as an active component of such preparations (21) although it cannot of itself entirely account for lipocaic activity (22). Further, we are now determining the effects of inositol and tocopherol on the deposition of ceroid in cirrhotic livers due to choline deficiency.

This work was supported by grants from the Josiah Macy, Jr., Foundation, the Duke University Research Council, and a Charles B. Mayer fellowship of the New York Academy of Medicine. Our thanks are due to Dr. Thomas L. Jukes and Dr. E. L. R. Stokstad of the Lederle Laboratories, Inc., for their generous gift of folic acid, to Merck and Company, Inc., for the other crystalline vitamins used in this work, and to Dr. Dean Burk for a biotin analysis of the liver extract.

SUMMARY

The administration of biotin plus a basal B vitamin supplement to rats which had previously been fed a low protein diet with no B vitamins resulted in fatty livers despite the presence of adequate amounts of dietary choline. This was accompanied by a considerable increase in food consumption and growth. The further addition of folic acid accentuated these phenomena, although folic acid without biotin did not produce these effects. Inositol prevented the accumulation of liver fat without affecting either appetite or the growth rate. When a relatively high fat diet plus the basal B vitamin supplement was given to previously depleted rats, inositol was again found necessary to maintain normal liver lipid concentrations even in the absence of supplementary biotin, folic acid, or liver extract, and the effects of biotin plus folic acid were even more pronounced on this diet. However, if the experiments were continued for 24 days, choline alone sufficed to provide normal liver fat concentrations, as the food consumption and growth rate declined after the 1st week with a consequent decrease in the demand for dietary lipotropic factors to a level at which the supply of inositol from synthesis by the intestinal flora was sufficient to meet the inositol requirement. When sulfasuxidine was included in both the depletion ration and the experimental diet, the effects of the biotin-folic acid combination were again manifest but neither alone markedly affected appetite, growth, or liver lipid concentrations. However, the demand for dietary inositol was still apparent even after 24 days, presumably because of inhibition of intraintestinal synthesis. The inhibition of the lipotropic action of inositol by unsaturated fatty acids was confirmed and suggestive evidence was obtained for a synergistic activity of inositol and tocopherol in this system, although tocopherol of itself exerted no apparent lipotropic activity. The significance of these findings is discussed.

BIBLIOGRAPHY

1. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **134**, 683 (1940).
2. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **141**, 619 (1941).
3. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 485 (1941).
4. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940).
5. MacFarland, M. L., and McHenry, E. W., *J. Biol. Chem.*, **159**, 605 (1945).
6. Wolfe, J. M., Bryan, W. R., and Wright, A. W., *Am. J. Cancer*, **34**, 352 (1938).
7. Hubbell, R. G., Mendel, L. B., and Wakeman, A. J., *J. Nutr.*, **14**, 273 (1937).
8. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **146**, 357 (1942).
9. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
10. Beveridge, J. M. R., and Lucas, C. C., *J. Biol. Chem.*, **157**, 311 (1945).
11. Handler, P., and Bernheim, F., *J. Biol. Chem.*, **148**, 649 (1943).
12. Handler, P., *J. Biol. Chem.*, **149**, 291 (1943).
13. Handler, P., *J. Biol. Chem.*, **154**, 203 (1944).
14. Milhorat, A. T., and Bartels, W. E., *Science*, **101**, 93 (1945).
15. Dam, H., and Glavind, J., *Science*, **96**, 235 (1942).
16. Dam, H., *J. Nutr.*, **27**, 193 (1944).
17. Lillie, R. D., Ashburn, L. L., Sebrell, W. H., Daft, F. S., and Lowry, J. V., *Pub. Health Rep., U. S. P. H. S.*, **57**, 502 (1942).
18. Endicott, K. M., *Arch. Path.*, **37**, 49 (1944).
19. Wachstein, M., *Proc. Soc. Exp. Biol. and Med.*, **59**, 73 (1945).
20. Mason, K. E., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **2** (1944).
21. Abels, J. C., Kupel, C. W., Pack, G. T., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, **54**, 157 (1943).
22. Owens, F. M., Jr., Allen, J. G., Stinger, D., and Dragstedt, L. R., *Federation Proc.*, **1**, pt. 2, 65 (1942).

J. Nutr. 31:621-633, 1946

FACTORS AFFECTING THE OCCURRENCE OF HEMORRHAGIC KIDNEYS DUE TO CHOLINE DEFICIENCY

PHILIP HANDLER
Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina

(Received for publication December 3, 1945)

The hepato-renal syndrome of choline deficiency is now a well recognized entity in the albino rat. While fatty infiltration of the liver with its sequelae of necrosis and cirrhosis may be produced in rats of any age, renal lesions have only been observed consistently when weanling rats were offered diets which were choline free and of low methionine content but adequate in all other respects. Cholesterol, cystine and a high fat intake increase both the incidence and severity of the disease. This situation has been amply reviewed by Griffith ('41). There are also a few references to such lesions in older animals but these have not been consistent. From the work of Griffith it has seemed that such lesions are most likely to occur at that time when the rate of growth of the animal is at a maximum and when the phospholipid turnover in the kidney is greatest. These concepts have been substantiated by the work of Engel and Salmon ('41) and of Patterson, Keevil and McHenry ('44). The present paper records some observations concerning the effects of other dietary factors and lipotropic substances on the production of such lesions in young rats and the occurrence of these lesions in older rats.

EXPERIMENTAL

All the rats used in these studies were males of the Vanderbilt strain (Wolfe et al., '38). After weaning they were grown to a weight of 50 gm on a commercial stock chow and then transferred to the experimental diets. They were housed either in separate cages or in "group" cages, 4 rats per cage. Unless otherwise stated, all rats which did not die earlier were killed by decapitation on the eighth experimental day. Liver fat determinations were performed in the manner previously reported (Handler and Dann, '42). A few kidneys from each group were bisected and fixed in formalin, then sectioned and stained with hematoxylin and eosin. Complete histopathological descriptions will not be presented since the lesions, when found, so closely resembled those described by Christensen ('42).

Food consumption was measured daily. However, the figures in the table were calculated only for the first 5 days on the experimental diet since many animals, on becoming ill on the sixth day or later completely stopped eating and comparison of food intake for the entire experimental period with animals which did not show kidney lesions would not be valid. The food intake for rats in group cages was, of necessity, calculated as one-quarter of the food disappearing in each group cage. All values in the tables are expressed in terms of one rat for the 5-day period.

The various diets used are summarized in table 1. The additions noted in table 2 were actually substituted for an equivalent amount of the dietary carbohydrate. In addition all diets except K_{10} contained, in mg per kilo, thiamine hydrochloride 5, riboflavin 10, calcium pantothenate 40 and pyridoxine 3.

The system of grading kidney lesions requires some explanation. One plus (+) indicates lesions in only one kidney, usually only a slight mottling and almost invariably this was in the right kidney. Two plus (++) indicates some gross lesions in both kidneys. Three plus (+++) indicates the full blown disease with two, large, hyperemic, almost spleen colored kidneys while four plus (++++) has been used to indicate the existence of hemorrhage extending beyond the kidney and into the dorsal wall of the peritoneum. This system is crude but was quite helpful in comparing the effects of different dietary regimes.

TABLE 1

Composition of diets.[1]

	K_1	K_2	K_3	K_4	K_5	K_6	K_7	K_8	K_9	K_{10}	K_{11}	K_{12}	K_{13}
Casein	15	15	15	15	15	10	8	6	4	10	12	10	12
Sucrose	58	—	—	—	—	—	—	—	—	84	71	67	57
Starch	—	—	58	—	—	63	65	67	69	—	—	—	—
Glucose	—	58	—	—	—	—	—	—	—	—	—	—	—
Lactose	—	—	—	58	—	—	—	—	—	—	—	—	—
Galactose	—	—	—	—	58	—	—	—	—	—	—	—	—
Cotton oil	15	15	15	15	15	15	15	15	15	—	—	15	15
Salts[2]	5	5	5	5	5	5	5	5	5	4	4	4	4
Crisco	—	—	—	—	—	—	—	—	—	—	10	—	5
Cod liver oil	5	5	5	5	5	5	4	4	4	—	—	4	5
Cholesterol	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0.5	0.5	—	0.5	—	1.0
Cystine	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	—	0.4	—	0.5
Cellulose	—	—	—	—	—	—	—	—	—	2.0	2.0	—	—
Sulfasuxidine	—	—	—	—	—	—	—	—	—	1.0	1.0	—	—

[1] The additions noted in table 2 were substituted for an equivalent amount of dietary carbohydrate. In addition all diets except K_{10} contained, in mg/kg, thiamine 5, riboflavin 10, calcium pantothenate 40 and pyridoxine 3.

[2] Hubbell, R. G., L. B. Mendel and A. J. Wakeman, 1937, J. Nutrition, vol. 14, p. 273.

Production of hemorrhagic kidneys in young rats

When rats were housed in single cages the diet K_1, which was patterned after those described by Griffith ('41), proved most suitable for the production of kidney lesions. As shown in table 2, all rats on this basal diet showed some sign of kidney damage. However, when the animals were housed in group cages during the experimental period both the incidence and severity of the disease was markedly less than in the single cages, although liver fat concentrations and food intake were of the same magnitude in both groups. The only

explanation which has suggested itself is that the practice of coprophagy provided the rats in group cages with sufficient choline (or source of labile methyl groups) to protect the kidney against injury but not enough to reduce liver fat concentrations effectively. Evidence suggesting that some synthesis, presumably intra-intestinal, of such compounds does occur has recently been furnished by du Vigneaud et al., ('45).

In an attempt to increase the demand for methionine and therefore choline, nicotinamide was added to the diet at a concentration of 0.5%. The rationale for this procedure has

TABLE 2

Effects of various dietary factors on incidence of hemorrhagic kidneys when used as supplements to the basal ration K_1.

SPECIAL SUPPLEMENT	NO. RATS	FOOD INTAKE	LIVER FAT	HEMORRHAGIC KIDNEYS 4+	3+	2+	+	0	TYPE OF CAGE
		gm	%						
.....	24	40	14.7	1	15	7	1	0	Single
.....	24	36	16.0	0	0	6	8	10	Group
0.5% Nicotinamide	12	21	18.4	0	4	4	3	1	Group
0.5% Nicotinamide	12	22	20.2	0	2	4	3	3	Single
0.25% Inositol	24	37	12.8	17	6	0	1	0	Single
0.25% Inositol	12	34	11.6	1	7	2	1	1	Group
0.25% Inositol + 0.5% nicotinamide	24	22	13.1	8	7	5	3	1	Group
α-Tocopherol 500 μg/day	12	36	15.9	0	7	4	0	1	Single
α-Tocopherol 500 μg/day + 0.25% Inositol	12	38	9.7	0	2	3	3	4	Single
1% Lipocaic	24	35	9.1	0	1	7	5	9	Single
2% Meat extract	12	40	6.1	0	0	0	0	12	Single
5% Liver extract	12	39	5.4	0	0	0	0	12	Single
Biotin 20 μg/day + folic acid 20 μg/day	12	39	17.1	0	6	5	1	0	Single
0.15% Choline	24	41	8.7	0	5	4	8	8	Single
0.25% Choline	24	42	4.8	0	0	3	6	15	Single
0.5% Nicotinamide + 0.35% methionine	12	30	7.7	0	1	2	4	7	Group
0.25% Choline + 0.5% nicotinamide	24	26	11.4	0	7	2	8	8	Group
0.5% Choline + 0.5% nicotinamide	24	29	6.1	0	0	0	2	22	Group
Choline 6 mg/day + 0.25 inositol	12	37	8.7	2	3	4	1	2	Single

been discussed by Handler and Dann ('42). This resulted in a considerably increased incidence of hemorrhagic kidneys in the rats in group cages but actually diminished the number seen in single cages. The latter effect appears to have been due to the diminution of food intake caused by the nicotinamide induced methionine deficiency.

Effects of various lipotropic and alipotropic factors

The effects of various materials which have been suggested as having lipotropic or alipotropic activity were then tested. The results are also included in table 2. While somewhat reducing the liver fat concentration, inositol actually appeared to increase the incidence and severity of the renal syndrome. In many inositol fed animals the gross hemorrhage which is primarily intra and subcapsular appeared to spread out so that the entire dorsal wall of the peritoneum appeared hemorrhagic. Such animals also invariably showed hemorrhage in the pericardi[illegible], lungs and eyesockets as well. These extensive lesions were seldom seen on diets which did not contain inositol.

Tocopherol deficient chicks exhibit capillary damage which is manifested as a generalized exudative diathesis which is, however, prevented by the administration of inositol (Dam and Glavind, '42). Of itself, tocopherol exerted no activity in the present experiments. Tocopherol plus inositol, however, significantly decreased the incidence of hemorrhagic kidneys below that seen in the control groups, in striking contrast to the action of inositol alone. This combination also exerted significant lipotropic action in the liver. Since inositol has been implicated as an active component of "lipocaic" preparations (Abels et al., '43; Gavin and McHenry, '41 a), it was thought of interest to test its activity against choline deficiency hemorrhagic kidneys. Lipocaic was prepared by the method of Clark et al. ('39) from frozen beef pancreas obtained commercially and was included as 1% of the diet. At this level the preparation proved quite efficacious in preventing hemorrhagic kidneys. Unfortunately, the choline con-

tent of the preparation, determined by colorimetric estimation of the reineckate, permitted a daily intake of about 1.8 mg of choline. This level of choline plus 0.25% inositol had relatively little effect on the incidence of renal lesions. However, it cannot be stated whether the activity of the lipocaic was a direct one on the kidney, a sparing action in the liver or, perhaps, due to more complete liberation and availability of the methionine in the dietary casein (Chaikoff et al., '45). We are now attempting to obtain a choline free preparation of lipocaic to test it in this manner. It should be noted that the lipocaic also effectively prevented the accumulation of liver fat as well.

The fatty livers of depancreatized dogs maintained on a diet of lean beef and sucrose do not occur when the insoluble residue from the preparation of commercial meat juice extract are fed, but are manifested when the meat extract is included in the diet (Ralli and Rubin, '42). It seemed of interest to test the effect of meat juice by the present technique and it was found that when meat extract [1] was included as 2.5% of the diet only normal livers and kidneys were found. However choline analysis revealed that the preparation was providing about 17 mg of choline per day which, even in the presence of the inositol may well have been sufficient to account for the results.

Because of the suggestion that biotin increases the demand for lipotropic factors (Gavin and McHenry, '41 b) the effects of biotin,[2] crystalline folic acid [3] and a liver extract prepared according to the directions of McHenry and Gavin ('40) were tested. Neither biotin nor folic acid nor a combination of the two appeared to have any effect. The liver extract, rather than increasing the demand for lipotropic factors, actually prevented the appearance of hemorrhagic kidneys. This again was undoubtedly due to its choline content as it provided at least 11 mg of choline and an unknown amount of methionine

[1] Valentine's.
[2] Merck.
[3] Lederle.

per day. The actual roles of biotin, folic acid and inositol in the homeostatic control of liver fat concentrations has been discussed elsewhere (Handler, '46).

Influence of the dietary carbohydrate on the incidence of hemorrhagic kidneys

Griffith ('41) has stated that starch used in place of sucrose in synthetic diets prevented the appearance of hemorrhagic kidneys although no data were presented illustrating the point. We have tested the effects of substituting starch, glucose, lactose and galactose for the sucrose of diet K_1. In contrast to Griffith's statement both glucose and starch were found to be effective in such diets. When lactose or galactose were

TABLE 3

Effects of various carbohydrates in choline deficiency. All rats were housed in single cages.

DIET	CARBOHYDRATE	NO. RATS	FOOD INTAKE	LIVER FAT	HEMORRHAGIC KIDNEYS				
					4+	3+	2+	+	0
			gm	%					
K_1	Sucrose	24	40	14.7	1	15	7	1	0
K_2	Glucose	12	38	13.9	0	7	2	3	0
K_3	Starch	12	37	15.5	0	6	3	2	1
K_4	Lactose	12	26	7.4	0	0	0	1	11
K_5	Galactose	12	24	8.1	0	0	0	0	12

used no kidney lesions were found and further, there was only a relatively small increase in liver fat concentration. The daily food consumption was lower on these two diets, and it seems possible that this may account for these effects. While it is not shown in the data (table 3), those rats which ate most and did gain weight showed the largest liver fat concentrations. No rats on these diets ate or grew as well as those on the other diets. The general nutritional inadequacy of lactose as the sole carbohydrate in the diet of the rat has already been reported by Ershoff and Deuel ('44) who found that young rats die after 2 to 3 weeks on such diets. An attempt to determine the underlying mechanism of this nutritional failure will be described in a subsequent report.

Delayed appearance of hemorrhagic kidneys

While attempting to determine the optimal dietary conditions for the production of dietary hepatic cirrhosis, studies were made in which various levels of casein were tried (Handler and Dubin, '46). The diets used are summarized in table 1. At casein levels of 15, 10 and 8% (diets $K_{1, 6, 7}$) the incidence of deaths due to kidney damage between the sixth and thirteenth day were 65, 40 and 25% of the total number of animals, respectively. No animals died in this manner after the thirteenth day although they were maintained for 120 days on the same diets. When casein was fed as 6% of the diet, (K_8) no deaths occurred during the apparently critical period found in the series above. Surprisingly however, 17 out of 24 rats then died between the thirty-fourth and forty-fifth day of the experiment with acutely hemorrhagic kidneys. The survivors were sacrificed after 120 days at which time all but one were found to have the characteristic gross "frosted" kidneys indicative of kidney lesions which had healed and this was confirmed by examination of the sections. A second group of 24 animals were placed upon the same diet and groups of 6 were sacrificed at weekly intervals. No kidney lesions were noted in any but the last group sacrificed (28 days). It should also be added that no lesions were found when 4% casein was fed (K_9).

A delayed development of renal damage due to choline deficiency has also been noted under other conditions. These have been described elsewhere (Handler, '46) and so merit only brief mention in the present report insofar as is pertinent to the discussion. Male rats were fed diet K_{10} which contained 10% casein and no fat or B vitamin supplement for 21 days after they had been grown to a weight of 80 gm on a stock ration. They were then transferred to diet K_{11} which contained 12% casein, 10% crisco and adequate B vitamins including biotin and folic acid and sacrificed after 8 days. Nine out of 12 animals showed varying degrees of renal damage and all showed markedly fatty livers. Choline alone

was sufficient to prevent the renal lesions but did not quite produce normal liver fat concentrations. Inositol plus tocopherol also reduced the incidence of the kidney lesions and also considerably diminished the liver fat concentrations. While this appearance of hemorrhagic kidneys is not delayed in the same sense as those in the preceding experiment, it does again demonstrate that there is no critical age for the development of such lesions, but rather only a critical situation which may be produced or occur at any age.

Hemorrhagic kidneys in unilaterally nephrectomized rats

The renal lesions of choline deficiency have only occasionally been noted to develop in mature rats. To check this, diets K_1 (15% casein) and K_7 (8% casein) were each offered to a group of 12 rats after they had attained a weight of 250 gm on a stock ration. The animals were sacrificed after 100 days. Four of the rats on K_7, exhibited grossly cirrhotic livers and all livers in both groups were extremely fatty although quantitative estimations were not performed. All the animals on K_1 were found to have normal kidneys and 3 rats on K_7 appeared to show slight renal damage.

Forty adult male rats, all of which weighed more than 250 gm were then fed diet K_{12} (10% casein) for 6 weeks to deplete them of any choline or labile methyl reserves. They were then subjected to unilateral nephrectomy under ether anesthesia. In each case, the right kidney was removed as this is normally slightly larger than the left kidney (see table 4). They were then changed to diet K_{13} (12% casein, 20% fat) and groups of 10 were sacrificed after 8, 10, 12 and 14 days. The results and comparison with control animals on a stock regime are shown in table 4. It will be seen that hemorrhages were noted in all groups but the critical period appeared to lie between the tenth and fourteenth day after nephrectomy. Actually 4 rats were found dead on the thirteenth day with severe renal lesions and are included in the table with the group sacrificed on the fourteenth day. The

large size of the kidneys noted in these rats was undoubtedly due to a combination of hypertrophy and hyperemia such as that seen in choline deficient weanling rats on such diets.

As a control, 12 rats of the same size maintained on a stock ration were subjected to unilateral nephrectomy in the same fashion. Two weeks later they were placed on diet K_{13}. All rats survived for another 6 weeks when they were sacrificed. Moderate lesions were found in only one rat.

TABLE 4

Hemorrhagic kidneys in unilaterally nephrectomized rats. Each value is a mean for a group of 10 rats.

STATE OF RATS	DIET	TIME	LEFT KIDNEY WEIGHT	RIGHT KIDNEY WEIGHT	HEMORRHAGIC KIDNEYS			
					3+	2+	+	0
		days	*gm*	*gm*				
Normal	Stock	0	0.61	0.67	0	0	0	10
Right kidney removed	Stock	8	1.04	0.71	0	0	0	10
Right kidney removed	Stock	14	1.17	0.70	0	0	0	10
Right kidney removed	K_{13}	8	1.08	0.66	0	0	3	7
Right kidney removed	K_{13}	10	1.19	0.72	0	1	4	5
Right kidney removed	K_{13}	12	1.38	0.70	0	3	3	4
Right kidney removed	K_{13}	14	1.70	0.74	3	3	2	2

DISCUSSION

The data presented show that renal lesions due to choline deficiency may occur or be induced at any age and are most likely to occur when the food consumption, growth rate and lipid turnover are maximal. Ordinarily, the lipid turnover in the kidney probably parallels that in the entire animal. During the period of hypertrophy after unilateral nephrectomy in adult animals, however, it appeared that the demand for lipotropic factors was at least as great as in the young growing rat and when these were not provided in the diet the typical picture of choline deficiency renal damage was found in the remaining kidney. Further, renal damage due to choline deficiency appears to be an acute rather than a chronic cumulative process. The study of delayed appearance of such lesions on the 6% casein diet (K_8) confirmed this concept with regard to the morphological aspect of the kidneys, yet demonstrated

that such damage may occur at any time during the life cycle if proper conditions exist, although it is difficult to state precisely what these conditions are.

Despite its lipotropic action in the liver, inositol did not afford any protection against renal damage, but, if anything, appeared to increase both the severity and incidence of the disease. On the other hand a combination of inositol and tocopherol offered some protection. A lipocaic preparation also appeared to offer a greater degree of protection than was warranted by its choline content. It cannot be said whether this was due to a combination of factors or to an actual chemical entity which has been designated as lipocaic. If the preparation contained an appreciable amount of tocopherol and inositol this might have been responsible for the observed activity. This would parallel the inositol-tocopherol relationship in preventing the capillary damage and exudative diathesis of vitamin E deficiency (Dam and Glavind, '42). Dam ('44) has stated that no tocopherol was found in an alkaline hydrolysate of a lipocaic preparation but it is doubtful that any tocopherol could have remained after such drastic treatment nor would it necessarily have split an inositol-tocopherol ether such as that proposed by Milhorat and Bartels ('45).

SUMMARY

Rats housed in group cages failed to develop the renal lesions of choline deficiency on a diet which was quite effective when the rats were in single cages. The addition of nicotinamide to this diet resulted in renal lesions even in the rats housed in group cages. While inositol exerted lipotropic activity in the liver, it appeared to increase slightly the incidence of hemorrhagic kidneys due to choline deficiency. A combination of inositol and tocopherol as well as a lipocaic preparation significantly decreased the incidence of such lesions while biotin and folic acid were without effect. Weanling rats on a diet containing 6% casein did not develop hemorrhagic kidneys until after 35 to 45 days, in contrast to rats receiving diets of higher protein concentration which

develop such lesions in 6 to 10 days. While few adult rats developed renal lesions on choline deficient rations, choline deficient adult rats subjected to unilateral nephrectomy uniformly showed such lesions after 10 to 14 days, the period during which the remaining kidney hypertrophied. When adult nephrectomized rats were placed on a choline deficient regime 2 weeks after the operation, renal damage was observed in only one of 12 animals although they were continued on the diet for 6 weeks.

ACKNOWLEDGMENTS

This work was supported by the Josiah Macy, Jr., Foundation, a Charles B. Mayer fellowship of the New York Academy of Medicine and the Duke University Research Council. Our thanks are due also to Merck and Company, Inc., for the crystalline vitamins used in the study, and to the Lederle Laboratories, Inc., for a gift of folic acid.

LITERATURE CITED

ABELS, J. C., C. W. KUPEL, G. T. PACK AND C. P. RHOADS 1943 Metabolic studies in patients with cancer of gastro-intestinal tract IV. Lipotropic properties of inositol. Proc. Soc. Exp. Biol. Med., vol. 54, p. 157.

CHAIKOFF, I. L., C. ENTENMAN AND M .L. MONTGOMERY 1945 The mechanism of action of the antifatty liver factor of the pancreas. II. Free methionine prevents fatty livers in completely depancreatized dogs maintained with insulin and fed a lean meat diet. J. Biol. Chem., vol. 160, p. 489.

CHRISTENSEN, K. 1942 Renal changes in the albino rat on low choline and choline-deficient diets. Arch. Path., vol. 34, p. 633.

CLARK, D. E., C. W. VERMEULEN, G. DONOVAN AND L. R. DRAGSTEDT 1939 The preparation of lipocaic. Am. J. Physiol., vol. 126, p. 464.

DAM, H. 1944 Studies on vitamin E deficiency in chicks. J. Nutrition, vol. 27, p. 193.

DAM, H., AND J. GLAVIND 1942 Factors influencing capillary permeability in the vitamin E chick. Science, vol. 96, p. 235.

DU VIGNEAUD, V. S. SIMMONDS, J. P. CHANDLER AND M. COHN 1945 Synthesis of labile methyl groups in the white rat. J. Biol. Chem., vol. 159, p. 755.

ENGEL, R. W., AND W. D. SALMON 1941 Improved diets for nutritional and pathologic studies of choline deficiency in young rats. J. Nutrition, vol. 22, p. 109.

ERSHOFF, B. H., AND H. J. DEUEL, JR. 1944 Inadequacy of lactose and beta-lactose as dietary carbohydrates for the rat. J. Nutrition, vol. 38, p. 225.

GAVIN, G., AND E. W. MCHENRY 1941 a Inositol: A lipotropic factor. J. Biol. Chem., vol. 139, p. 485.

———— 1941 b The effects of biotin upon fat synthesis and metabolism. J. Biol. Chem., vol. 141, p. 619.

GRIFFITH, W. H. 1941 The nutritional importance of choline. J. Nutrition, vol. 22, p. 239.

HANDLER, P. 1946 Dietary factors in the regulation of liver lipid concentration. J. Biol. Chem., vol. 162, p. 77.

HANDLER, P., AND W. J. DANN 1942 The inhibition of rat growth by nicotinamide. J. Biol. Chem., vol. 146, p. 357.

HANDLER, P., AND I. N. DUBIN 1946 The significance of fatty infiltration in the development of hepatic cirrhosis due to choline deficiency. J. Nutrition, vol. 31, p. 141.

MCHENRY, E. W., AND G. GAVIN 1940 The effects of liver and pancreas extracts upon fat synthesis and metabolism. J. Biol. Chem., vol. 134, p. 683.

MILHORAT, A. T., AND W. E. BARTELS 1945 Defect in utilization of tocopherol in progressive muscular dystrophy. Science, vol. 101, p. 93.

PATTERSON, J. M., N. B. KEEVIL AND E. W. MCHENRY 1944 Choline and the prevention of hemorrhagic kidneys in the rat. II. Phospholipid turnover determined with radioactive phosphorus. J. Biol. Chem., vol. 153, p. 489.

RALLI, E. P., AND S. H. RUBIN 1942 The effect of meat and meat fractions on the fatty liver of the depancreatized and pancreatic-duct ligated dog. Am. J. Physiol., vol. 138, p. 42.

WOLFE, J. M., W. R. BRYAN AND A. W. WRIGHT 1938 Histologic observations on the anterior pituitaries of old rats with particular reference to the spontaneous appearance of pituitary adenomata. Am. J. Cancer, vol. 34, p. 352.

Proc. Soc. Exptl. Biol. Med. 63,436-438, 1946

15627 P

Inositol Decholesterization in Old Hens.*†

GEORGE R. HERRMANN.

From the Department of Internal Medicine, University of Texas.

Inositol was found by Gavin and McHenry[1] to prevent the development of a special type of biotin fatty liver, but no such lipotropic activity was demonstrated against thiamin fatty livers. Abels *et al.*[2] found that inositol accounted for the lipotropic property of a lipocaic preparation when given to patients with gastrointestinal cancer. Inositol administered preoperatively, reduced the average concentration of fat to 46% of that of almost uniformly fatty infiltrated livers of untreated patients. Shay[3] could demonstrate no reduction in the size of fatty livers in diabetics nor any changes in the blood cholesterol by the daily administration of 1.2 g of inositol. However, Russakoff and Blumberg[4] reported reduction in the blood cholesterol and cure by inositol in doses of 1 g per day, in a patient with a dermatological condition associated with a disturbed fat metabolism.

* With the technical assistance of Anna H. Williams, Lucy Prosise, H. Tom Leigh, John Prewett, and George W. Reimer.

† Inositol was generously supplied and the study was, in part, supported by a grant from the Medical Research Department of the Winthrop Chemical Co.

1 Gavin, G., and McHenry, W. E., *J. Biol. Chem.*, 1941, **139**, 485; 1943, **148**, 25.

2 Abels, J. C., Kupel, C. W., Pack, C. T., and Rhoads, C. P., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 157.

3 Shay, H., *Am. J. Digest. Dis.*, 1943, **10**, 48.

4 Blumberg, H., and Russakoff, A. H., unpublished data, quoted in *Ann. Int. Med.*, 1944, **21**, 848.

TABLE I.
Effect of Inositol on Blood and Tissue Lipids.

	Blood cholesterol, mg/100 ml		Phospho-lipids	Tissue Cholesterol, mg/100 g					
				Aorta		Heart		Liver	
	Total	Esters	mg/100 ml	Total	Esters	Total	Esters	Total	Esters
Controls	274.8	166	9.6	230	176.2	275	223	340	240
36 old hens S.D.	±39	±19	±2.7	±41	±20.4	±71	±19	±65	±24
(A) Series (7)									
Inositol 1st Bl.*	254 ±28	201±29	7.5±1.7						
25-30 2nd Bl.	220 ±29	169±26	10 ±1.1						
days 3rd Bl.	202 ±18	151±16	9.4±0.7	210	160	172	126	281	221
				±10	±19	±9.6	±9	±28.5	±23.5
(B) Series (16)									
Ino.† control									
1st Bl.	230 ±29	181±27	8.6±1.3						
Ino. 25 days									
2nd Bl.	206 ±20	160±20	11.3±1.9						
Ino. 55 to 68 days				197	145	167	127	241	178
3rd bl.	176 ±13	140±12	12.9±1.9	±27	±22	±40	±29	±60	±35
(C) Series (23)									
Inositol 1st Bl.	237 ±29	187±31	8.7±1,2						
2nd Bl.	210 ±24	163±20	11 ±1.9						
30-68 days 3rd Bl.	184 ±19	135±16	11.8±1.9	201	155	166	129	253	191
				±38	±24	±37	±29	±55	±93

* Bl.—Bleeding. †Inositol. S.D.—Standard Deviation, ±.

Further studies seemed to be in order and inositol was therefore subjected to animal experimental studies similar to those made with other lipotropic substances.[5]

Two series of old hens from high egg-producing stock were individually caged and given the standard high-fat laying mash (Purina). Blood was withdrawn from 16, and later 7 more, hens for the determination of control levels of total cholesterol, cholesterol esters, total and inorganic phosphorous levels by the methods of Bloor,[6] Bloor and Knudson,[7] and King,[8] respectively.

Inositol, 0.5 g was then added to the diet, and put down the gullet of each hen daily. All the birds were healthy throughout the experiment. After 25 days blood was again removed for the chemical studies and at the sacrifice after 30 days in 7 hens and after 62 days in 16 hens. The aorta and approximately 0.25 g of heart muscle and a similar mass of liver were removed, weighed accurately and macerated with sand and extracted with ether alcohol. The 10 cc extract of each organ was analyzed and the amount of total cholesterol and cholesterol esters in 100 g of each tissue calculated. The statistically-treated data for the 7 hens treated for 25 to 30 days and for the 16 hens treated for 55 to 68 days are set down in Table I and for all 23 inositol-treated hens for comparison with the normals previously established.[5]

It is evident that barely significant reductions in cholesterol in blood and tissues develop in 30 days while definite reductions have been recorded in 55 to 68 days.

Under the conditions of this experiment, the total blood cholesterol dropped about 11% and the cholesterol esters about 13%, but the organic or lipid phosphorous rose about 31% after 25 days of inositol. After 55 to 68 days, the average total blood cholesterol had dropped 22%, the esters 17%, and the phospholipids had risen 50%. The tissue analysis of inositol-treated hens showed reductions in concentration of 9% and 9% of cholesterol total and esters in the aorta in 30 days and 14% and 12% in 52 to 68 days. The heart muscle levels dropped 37.5% and 43% in 30 days and 40% and 41% in 55 to 68 days, and those in the liver were 17.6% and 18.3% in 30 days and 25% and 20% in 55 to 68 days respectively below the levels in the controls established in previous studies.[8]

The results seem to indicate that inositol is a decholesterizing agent and effects some mobilization of cholesterol and cholesterol esters from the tissues.

[5] Herrmann, G. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 229 and 302. (Note: In these two communications the subheading under Tissue Cholesterol total/esters determinations on aorta, heart, and liver were erroneously set down as mg/250 mg instead of mg/100 g to which amounts the calculations had been made.)

[6] Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

[7] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

[8] King, E. J., *Biol. Chem.*, 1932, **26**, 240.

Record of Chemical Progress

An Official Organ of Section C of the American Association for the Advancement of Science

Published by
THE FRIENDS OF THE KRESGE-HOOKER SCIENTIFIC LIBRARY
Editor, Neil E. Gordon; Associate Editor, Mary Elvira Weeks
Wayne University, Detroit 1, Michigan

Volume 5 **July-October, 1944** **Nos. 3 and 4**

Cancer

Chemotherapeutic Regression of Malignant Mouse Tumors

MARIE L. HESSELBACH and DEAN BURK, *National Cancer Institute, Bethesda, Maryland*

AUTHENTICATED cases of complete regression of *spontaneous* malignant tumors in either man or animals are rare. Demonstrated causation of such regressions by known chemotherapeutic agents has, until recently, been virtually unknown or without general acceptance. A group of investigators* at the Cancer Research Laboratory of the Mt. Sinai Hospital in New York City are now reporting the accomplishment of this object in several strains of mice, upon injection of pure chemicals at sites removed from the cancers (remote administration). This new research is of unusual interest to experimental cancer workers, clinicians and industrial biochemists alike.

For eight years, the Mt. Sinai group have carried their work (1–15a) through a logical development from early pioneering experiments, in which various non-specific tissue extracts and techniques were explored, to current ones in which a combination of well-established methods is used to test definite chemical entities. In brief, studies have been made on inhibiting effects produced by extracts of various animal tissues, yeasts and grains, and of the B vitamins, on transplanted mouse Sarcoma 180 and spontaneous mammary adenocarcinomas of Bar Harbor Strain A and Rockland Farms mice, the agents usually being introduced by intravenous injection, and sometimes accompanied by dietary modifications. The most recent experiments are the most arresting, and are concerned with regressions produced by folic acid and related substances (folic acid vitamers (16)), and other known vitamins.

Beef-Spleen Extracts.—The first report, published in 1938 (1), was concerned chiefly with effective spleen extracts, and was based upon the clinical observation that carcinomatosis of the spleen is relatively rare. It was demonstrated that earlier workers who obtained negative results with spleen extracts were unsuccessful possibly because the dosages employed were too small. Very low doses were found, in fact, to be definitely stimulating to tumor growth (1), and often caused Sarcoma 180, which is normally nonmetastatic, to produce intraperitoneal and other metastases.

The first spleen extracts prepared were examined for their power to cause regression of actively growing *transplanted* mouse tumors (Sarcoma 180, obtained from the Crocker Laboratory). The mice used were healthy Rockland Farms females, weighing 18 to 20 gm. Each tumor was tested before transplantation, and some afterward, for microbiological sterility, and heart blood tests for paratyphoid organisms were also made on a percentage of the mice. Aqueous sterile extracts of beef spleen, equivalent to 100 gm. of tissue per cc., were injected subcutaneously near the left hip joint. There was no irritation, inflammation or infection at the site of injection. The first injection was given 7 to 9 days after the tumor transplant was made. Of 281 mice treated with 0.5-cc. doses of spleen extract—the only organ with any important potency—there were 170 cases, or 60%, of complete tumor regression; in the control group of 290 mice, there were 23 spontaneous regressions, or 8%. Although the active spleen extract employed did not show the Shwartzman skin-reaction phenomenon, it did cause tumor hemorrhage in much the same manner as do Shwartzman-positive preparations. The initial hemorrhage appeared anywhere from several hours after the first injection up until 3 or 4 daily injections had been given. When the hemorrhage had increased in size until it was surrounded by only a thin wall of fresh tumor tissue, the tumor decreased or stopped its growth, and a scab covered the hemorrhagic area. Later, this scab with underlying necrotic tumor was expelled, leaving a large cavity which broke into the remaining tumor tissue ring and absorbed it all. After a few weeks of daily injections, the tumor disappeared completely, and the area was absolutely normal except for a slight alopecia. It took 2 to 3 weeks of treatment for moderate-sized tumors to disappear and 4 to 6 weeks for large ones. Injections had to be continued for 2 or 3 weeks after there was no outward evidence of residual tumor. Excellent care had to be taken of these animals, and the frequency of injection individualized, to cut down mortality. No gross recurrence of these tumors occurred within a five-month period of observation. Most of the cured animals dying of natural causes during this period had no traces of tumor-remnants on histologic examination. The same spleen extract was also tried on a rat sarcoma and a few spontaneous breast tumors of mice, without effect (biopsies were not performed).

Healed-Spleen Extracts.—It was next reported (1, 2) that the spleens of the mice cured of their tumors by beef-spleen injections were enlarged

* This group consists at present of Dr. Richard Lewisohn, Cecilie Leuchtenberger, Dr. Rudolf Leuchtenberger and Dr. Daniel Laszlo, and formerly consisted of Dr. K. Bloch and Dr. Z. Dische also. The work has been supported in part by the International Cancer Research Foundation, the New York Foundation, Merck & Co., Inc., and the Lederle Laboratories, Inc.

to 4 to 5 times normal size. This was regarded as suggestive of an indirect mode of action of the extract, operating by increasing the mouse's normal defense against the growth of tumors. Extracts were prepared from these enlarged spleens to contain the equivalent of 1 gm. of fresh tissue per cc. The mode and locale of injection were changed from subcutaneous on the leg to intravenous into the tail. The longer the period up to a point (8-12 weeks) between cure and autopsy, the more effective, seemingly, was the extract. It was thought possible that this might indicate an accumulation of antibodies in the spleen, following the regression of the tumor, as it is well known that the peak of antibody production may sometimes occur a few months after treatment. The mode of regression brought about by healed-spleen extract was somewhat different from that previously described. There was no hemorrhage during regression, but necrosis and mummification, or sequestration followed by healing by epithelialization. These extracts gave, in the Sarcoma 180-bearing mice, quicker cures and higher percentages thereof, than normal beef-spleen used at 100 times the concentration! They were the first extracts which proved successful in curing *spontaneous* breast tumors in mice.

Spontaneous Tumors and Spleen Extracts. In the next set of experiments (2), 85 Strain A mice, bearing spontaneous mammary adenocarcinomas, *all confirmed by biopsy*, received intravenous, and occasionally intraperitoneal or subcutaneous injections, but when used alone the last named was found ineffective for these tumors. Whereas Dr. C. C. Little had reported that spontaneous regressions of spontaneous malignant tumors, proved by biopsy, had never been observed in this strain of mouse, 33 animals treated as indicated with dialyzed concentrated beef-spleen extract gave 7 complete regressions (21%). Thirty-eight animals given the healed-spleen extract showed 13 such cures (34%). The remaining 14 mice were treated with "immune spleen extract" with 5 cures (35%); the "immune spleen extract" came from the spleen of a young bull that had received many subcutaneous inoculations over a period of months of tumor tissue from spontaneous breast cancers of Strain A mice and from human cancers. There was a total of 25 complete regressions in the three groups (30%). The tumors became edematous and gradually shrank. There were 8 eventual recurrences in these groups, which the investigators ascribed to too early discontinuance of injections.

By 1941 (5), 712 biopsies had been performed on Strain A mice bearing spontaneous tumors. The importance of making biopsies of spontaneous tumors, an established procedure in all of this work, was made evident by the finding that 99, or 13%, of the tumors examined proved to be nonmalignant or doubtful cases (adenomas, milk cysts, swollen glands, chronic inflammations). In answer to the criticism that biopsy, per se, might cause regressions, 81 Strain A mice were subjected to biopsy only, without chemotherapeutic treatment. Some tumors showed a marked reduction in size immediately following biopsy, due to evacuation of liquid. Others showed temporary cessation of growth, or temporary

recession, at varying times during the observation period. Such transient regressions also occurred spontaneously without biopsy or treatment. Permanent spontaneous regressions occurred, without known treatment, to the extent of less than 1%.

Yeast Extracts.—Following reports that yeast in the diet sometimes retarded the growth of experimental tumors, splenic extracts were now replaced by yeast extracts (3). Daily or alternate injections of aqueous extracts, containing approximately the equivalent of 3 gms. of brewers' yeast per cc. were injected intravenously into the tails of a group of mice bearing spontaneous breast cancers and, in instances, also subcutaneously, as far as possible from the tumor site. To avoid recurrences, injections were continued for some time after complete regression. Twenty-two Strain A and 11 Rockland Farms mice bearing spontaneous mammary cancers, so treated, gave 5 and 3 cures, respectively. By the same treatment, 30% of cures were obtained (7) in another group consisting of Strain A, Strain R III, and old Rockland Farms mice bearing spontaneous mammary tumors, and Strain R III mice bearing the highly malignant transplanted Carcinoma 2163. Careful postmortem and histologic examinations failed to show any remains of tumor tissue. At the time of death the animals had been tumor-free for from 1 to 12 months.

It was found (1) that the tumor-inhibiting factor could be destroyed, or even a tumor-stimulating factor could be formed, by digestion with weak alkali. In a given preparation, the inhibitory factor disappeared parallel with the disappearance of the ability to support bacterial growth. The active principle was found (7) to be water soluble, comparatively thermostable at neutral pH, nonprotein in nature, not affected by nitrous acid, and precipitable by high concentrations of ethanol and phosphotungstic acid but not by lead acetate or silver nitrate. It was adsorbed by Fuller's earth and norite, but not by permutit. Efforts at elution were unsuccessful. Autolysis of the yeast cells prior to extraction caused loss of the active principle.

The causation by both spleen and yeast extracts of regression of malignant tumors, and the biological and chemical characteristics of the active principle obtained from yeast, all gave some indication of what this principle might be (1). From here on, the work narrowed down more and more to the testing of specific chemical substances indicated, chief of which, of course, were the B vitamins. Pyridoxin, pantothenic acid, and biotin, injected singly, had no inhibitory effect on spontaneous adenocarcinomas (7). Riboflavin or pantothenic acid injection in addition to the yeast extract, gave no better results than yeast alone. The yeast extract itself contained only negligible amounts of pantothenic acid, riboflavin, biotin, thiamin, and nicotinic acid.

A set of experiments was next performed to test whether the effect of yeast extract alone, or in combination with certain B vitamins, given by intravenous injection, could influence the percentage of takes of Carcinoma 2163 in Strain R III mice. This carcinoma gave 95 to 100% takes in the Mt. Sinai laboratory, and proved very resistant to treatment. To a group of

animals receiving pantothenic acid, yeast extract was given only for the first 10 days after transplantation. In the experiments involving thiamin or riboflavin, yeast extract was given for 10 consecutive days, and stopped on the day of transplantation. Yeast extract alone gave 20% non-takes; with pantothenic acid also added, it gave 47% non-takes; or with riboflavin added also, 62%. The addition of thiamin had no effect.

As the percentage of cures by means of yeast extracts could not be raised either by increasing the dosage or by repeated daily injections, it was decided to try the additional influence of a restricted diet (9). Polished rice and carrots were substituted for mouse pellets and carrots. Since the combination of this diet and injections of yeast extract definitely accelerated the healing process, it was not necessary to continue the diet until the tumors had disappeared entirely, and so the mice were rarely on this restricted diet for more than three weeks. The diet alone was ineffective in causing regression of the spontaneous tumors of R III and Rockland Farms mice. Forty-one Rockland Farms mice on the rice diet treated with yeast extract injections showed 63% complete regressions, averaging 20 days of treatment, as against 47 days with yeast alone. Ten R III mice, similarly treated, gave 4 regressions (40%). Neither yeast extract alone, nor the combined treatments, had any effect on the transplantable, highly malignant ("August" strain) rat Carcinoma R 2426, obtained from Dr. Eisen of Columbia University. The polished rice diet had no effect here when used in combination with inactive yeast extracts.

In this connection, it is noteworthy that the presence or absence of active principle in yeast extracts depended markedly upon the method of extraction, the brewers' mode of culturing of the yeast cells, and their source.

Rapid Growth Test.—In fulfillment of a need for a rapid test for tumor inhibitors, the following technique was evolved (10): 7 to 10 days after inoculation with tumor tissue, groups of 7 mice each are carefully matched as to tumor size, within the diameter 1.0 ± 0.2 cm., which, at this stage, indicates vigorous growth with but few spontaneous regressive changes. The animals are injected twice a day for two days—the controls getting saline, and the experimental animals the test substance. After 48 hours, the difference in mean terminal size and weight of the tumors in the various groups permits the detection of tumor growth inhibitors.

Extracts of 14 molds tested by the rapid method were all ineffective; as were also the following crystalline substances: thiamin, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid, ascorbic acid, histamine, ethylenediamine, methionine, and a vitamin mixture consisting of thiamin, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid, biotin and choline.

Inhibitors in Grains.—Work with yeast was shelved in 1943 (9) when unavoidable changes in brewing processes at hand eliminated the particular sources of yeast yielding active extracts. In the meantime, polished rice had been examined as a source of inhibitor substances. Standard doses of yeast extract were given to groups of mice on various diets. The results seemed

to indicate that the inhibiting effect of polished rice was not due to dietary deficiency or caloric restriction, but to the presence of a heat-labile tumor-inhibiting substance therein. Aqueous extracts of rice, corresponding to 2.5 mgms. of solids per 0.1-cc. dose, were as effective as 5.25-mgm. doses of the standard yeast extract, both sets of animals being kept on the normal pellet-carrot diet. Other grains (13) were tested for their dietary effect on animals getting standard doses of a yeast extract. Pearled barley and soya beans gave increased percentages of inhibition, whereas wheat flour, oats and corn meal had some effect. Wheat germ and malted barley were ineffective.

Since pearled barley extract was effective in the rapid growth test with transplantable tumors, it was then used in a long-term experiment with spontaneous tumors in Strain A and Swiss mice from the Jackson Memorial Laboratory, and Rockland Farms mice, in combination with a pearled-barley diet (11). There were 19 regressions in 46 mice used (41%), including some with quite large tumors.

Inositol and Related Compounds.—Inositol, and sodium phytate and lipositol (obtained from Dr. D. W. Woolley), showed marked inhibiting effects in the rapid growth test, the animals being kept on a polished rice diet for the experimental period of 48 hours. In as yet unpublished experiments it was found that the polished rice diet was not essential, but gave larger effects than did a normal pellet diet. The effectiveness was shown to depend on the dosage used (of the order of 0.1 mgm. for inositol) and subcutaneous and oral dosages of inositol were without effect, even at very high dosages (30 mgms.). Four hundred animals were used for the inositol tests. As this substance may be obtained in pure, crystalline form, it makes a good standard of reference for inhibitor tests. The following substances, also tested by the rapid method and with a polished rice diet for the test period, were ineffective as tumor inhibitors: *l*-inositol, inosose, thiamin, riboflavin, pyridoxin, nicotinamide, pantothenic acid, *p*-aminobenzoic acid, biotin and choline.

It is interesting to note a report by Williams (17) on the injection by Taylor of inositol into hatching eggs which were inoculated with tumor. Striking inhibitory effects on both the growth of the cancers and the development of the embryos were observed; on the other hand, no effect was obtained on the percentage of takes of a transplanted mammary carcinoma in mice upon adding inositol to a purina chow diet containing supplementary B vitamins. There have been several reports indicating not an anti-, but a pro-carcinogenic effect of inositol on the production of hepatomas induced by oral administration of *p*-dimethylaminoazobenzene to rats (Kensler, 17; 18, 19).

Folic Acid Vitamers.—The recent, and probably most definitive, aspect of the work concerns inhibition by folic acid vitamers (16), with animals maintained on a normal diet (14). A "folic acid concentrate," obtained from Merck and Co., was very effective in tumor inhibition at doses of 0.16 microgram given twice daily for two days to animals bearing Sarcoma 180, in the 48-hour growth test; results of experiments in progress with spontaneous tumors are awaited with interest. Crystalline "*L. casei*

factor," obtained from the Lederle Laboratories, produced at 0.025-microgram doses the same 48-hour test inhibition as did the "folic acid concentrate" at 0.16 microgram; 0.001 microgram produced a detectable effect.

In view of the tumor-growth inhibitory properties of the *L. casei* factor ("folic acid" vitamer), experiments are in progress in which mice bearing spontaneous breast adenocarcinomas are treated with this material. Though no definite conclusions can be drawn so far, the changes produced in these tumors are most striking. After a few intravenous injections some hard tumors are changed into soft bags containing necrotic tissue. Daily injections of 5 micrograms of *L. casei* factor were given, and 0.5- and 50-microgram dosages are also being tested, a total of about 175 mice having been so treated to date (September, 1943). In this work, it remains to be demonstrated whether, as in earlier work, other modes of administration than intravenous are ineffective; it would be our prediction that with adjustment of dosage other modes would be satisfactory.

Xanthopterin.—Xanthopterin, a pigment of yellow butterfly wings, and an antianemia factor for fish, has been reported by Wright and Welch (20) to be somewhat related to folic acid in activity. Synthetic xanthopterin (Merck and Lederle) was found (15) to be comparable in effectiveness to the "*L. casei* factor" in both the 48-hour growth test with transplantable tumors and the long-term regression of spontaneous breast tumor indicated above. On the other hand, leukopterin, the pigment of white butterfly wings, had no tumor-inhibiting effect in the 48-hour test, even at 100 γ. As the effective dose of xanthopterin was further increased, when used together with xanthopterin, it interfered with the effectiveness of the latter, acting as an antivitamer (16). In certain tests with rat liver, Wright and Welch (19) found xanthopterin, but not leukopterin, effective as a precursor of folic acid. It has been found that folic acid and biotin are relatively the most abundant of the B vitamins in rat and human tumors compared to normal tissues (Williams, 17). Perhaps it is significant that these two vitamins and some of their vitamers are among the most active of tumor inhibitors, but such possible significance remains to be clarified. Biotin and pyridoxin, which occur in the relatively smallest amounts in tumors generally (Williams, 17), have not caused tumor-inhibition in the present work. Work with xanthopterin is being shelved at present in favor of other folic acid vitamers, since it has been found that xanthopterin, at the dosages employed, eventually produces various types of secondary pathology in mice, and there appears to be an exceptional number of recurrences. Since the present research, like most researches, is limited by time and the resources at hand, it has been a pragmatic policy to spend little or no further effort on a material that gives indications of not being applicable clinically, regardless of whatever fundamental studies might be made further.

Discussion.—As regards the biochemical mode of action of the effective tumor inhibiting agents reported in this work, their action is usually to be distinguished from that of the bacterial filtrates or concentrates thereof (purified polysaccharide, endotoxic or O antigen, etc) that produce tumor or decidua-placental hemorrhage, the Shwartzman phenomenon, hyperthermia or fatality. All regressions, except those produced by the original beef-spleen extract (1), went through stages of necrosis or softening of tumor tissue, followed by expulsion or shrinkage, without hemorrhage; and none of the inhibitors yielded the Shwartzman skin-reaction, with the possible exception of one early, very toxic kidney extract (1).

An indirect, systemic mechanism, such as has been found for certain other vitamin and hormonal effects, seems indicated at present, possibly involving blood agents. Suggestive in this latter connection are: (*a*) Intravenous injections appear on the whole to be the most effective. (*b*) The known folic acid vitamers are active against anemia, leukopenia and granulocytopenia (20, 21, 22), and are thought to be involved in blood formation. (*c*) With certain of the inhibitors, involvement of the spleen is found (others remain to be studied). (*d*) Many well-known types of blood pathology are produced by the development and growth of cancers, some of which might conceivably be alleviated by folic acid in the instances where it produces regression.

A definite weakness in the experimental approach and direction of effort in the work being reported is the lack of studies on early histopathologic and biochemical changes during regression and recurrence, which, in the opinion of the present writers, would probably be much more valuable in the long run than all of the 48-hour growth test work, unless possibly the latter were vastly improved as to duration (1 to 2 weeks) and strain of transplant. Mixed tumor employed (one with few spontaneous regressions). Serial sectioning, rather than representative sectioning (at usually three depths) should be introduced, especially at initial and terminal stages of tumor changes, and in connection with distinguishing possible differences between chemotherapeutic and the rare spontaneous regressions, which differentiation is at present very difficult. The extensive use of the 48-hour test for tumor inhibitors, in spite of the authors' comment that comparisons should not necessarily be drawn between the effects here and in the longer-term experiments, probably rules out as many and more potential inhibitors than it detects, since complete regression undoubtedly involves many critical phenomena evident only after much longer periods of time. The test as reported could also be somewhat objected to on the grounds of lack of data on caloric and water intake and on the nature of the dietary régimes before, during and after the tests. Such data were indeed taken extensively, but were not described in detail since the investigators could detect no correlations of interest.

Accepting the great merit of the adopted final criterion of complete regression now in use, more emphasis should undoubtedly be given to histopathological and biochemical phenomena of partial regression, and also changes leading to complete regression, since these offer opportunity for a more fundamental understanding that is bound, in the long run, to yield the maximum therapeutic result.

Various esoteric features of the work need further clarification or experimental justification: the

claims of need for unbroken regularity of injection up to periods of months, in the long-term chemotherapy; for white instead of brown mice due to alleged vastly greater difficulty of continued tail injections (brown dba mice have, of course, light colored tails, though brown C3H mice do not); for intravenous rather than other modes of administration.

The scope of the work at present involves about 150 normal mice, of various derivations, a week. Routine testing of new groups of mice received, for paratyphoid organisms in blood specimens, is now carried out. To date about 6000 biopsies have been performed on spontaneous tumors, about equally divided between Strain A and Rockland Farms mice. No markedly different chemotherapeutic effects were noted between these two strains. About 20 Rockland Farms mice bearing spontaneous tumors are received each week, the mice being pen-bred and from a stock of low tumor incidence. The development of a high tumor-incidence strain of mice at the Mt. Sinai laboratory, now in its initial stages, would provide a dependable and sufficient supply of spontaneous tumors that could be observed from the time of origin. The rapid development of such a supply could be facilitated by treating large numbers of the readily available Strain A mice with stilbestrol, or Strain C with the milk factor. Extension of the work to male mice, and to tumors other than mammary adenocarcinomas, to other animals, to other folic acid vitamers, and to combinations of the latter with other vitamins, and to wider dosage ranges, would quite obviously be desirable.

The last, and probably most important recommendation that might be ventured, is that further efforts be made to have this work repeated in other cancer laboratories, and to have such results, whether positive or negative, published, or at least cited in the Chemotherapeutic Index.* To be sure, negative results can seldom have the significance and value of positive results in a field as complex as cancer chemotherapy, where rigid repetition is only an ideal; nevertheless, for what value they do possess, there is a definite obligation to make them known. The only published report on attempted repetition of the research described is the confirmatory work of Woglom (23) in 1940 with respect to the earliest phases involving extracts only.

Although much remains to be

* This is now being prepared under the direction of Dr. H. M. Dyer of the National Cancer Institute, following a suggestion made by Dr. Jacob Furth at the Gibson Island Cancer Conference in August, 1944 (17), that someone compile and publish all the data available relative to cancer therapy. Such a survey might be expected to aid other workers in planning systematic studies in this field of cancer research. All types of therapy except surgery and irradiation are to be listed, and negative as well as positive results of the treatment of spontaneous, transplanted and induced experimental tumors, and of clinical cases will be included. The data will be classified and tabulated in a simple manner similar to that in Hartwell's "Survey of Compounds Which Have Been Tested for Carcinogenic Activity," National Cancer Institute, 1941, and will include the name of the agent, the number of animals or patients treated, the dosage, route of injection, number of injections, the author's conclusions regarding a beneficial effect, the percentage of complete regressions if obtained, and the reference. Dr. Dyer will appreciate receiving both reprints of published work in this field, or any unpublished results that can be made available.

verified and made repeatable at will, the basic, experimentally supported claim of chemotherapeutically induced complete regressions of certain spontaneous mouse tumors is of undeniable importance and appears to be established. It is evident that extension of this work, in the laboratories of Mt. Sinai Hospital or elsewhere, should be watched with a good measure of interest.

REFERENCES

(1) Lewisohn, R., "Effect [illegible] cutaneous Injections of Co[illegible] Spleen Extract on Mouse Sarc[illegible] Surg., Gyn. and Obstet., *66*, 563[illegible]).

(2) Lewisohn, R., Leuc[illegible]er, R., and Laszlo, D., "Spleen Ex[illegible] in the Treatment of Transplanted [illegible] Spontaneous Malignant Tumor[illegible] Mice," *Ibid.*, *71*, 274–85 (1940).

(3) Lewisohn, R., L[illegible]berger, C., Leuchtenberger, R., a[illegible], D., "Effect of Intravenous Inj[illegible] Yeast Extract on Spontaneous [illegible] adenocarcinomas in Mice," Pr[illegible] Exp. Biol. and Med., *43*, 558–61[illegible]

(4) Lewisohn, R., Le[illegible]nberger, C., Leuchtenberger, R., and [illegible]szlo, D., "The Treatment of Spontaneous Breast Adenocarcinoma in Mice with Extracts of Spleen or Yeast," Am. J. Path., *17*, 251–60 (1941).

(5) Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., and Laszlo, D., "The Fate of Spontaneous Mammary Carcinomas in Mice after Simple Biopsy," Cancer Research, *1*, 324–7 (1941).

(6) Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., and Bloch, K., "Prevention of Tumor Growth (Carcinoma 2163) by Intravenous Injections of Yeast and Vitamins," Science, *94*, 70–1 (1941).

(7) Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., and Bloch, K., "Action of Yeast Extract on Transplanted and Spontaneous Malignant Tumors in Mice," Cancer Research, *1*, 799–806 (1941).

(8) Lewisohn, R., "Five Years of Cancer Research," J. Mt. Sinai Hosp., *8*, 771–6 (1942).

(9) Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., and Dische, Z., "Influence of a Polished Rice Diet upon Spontaneous Mammary Cancers in Mice Treated with Yeast Extract," Cancer Research, *2*, 818–22 (1942).

(10) Laszlo, D., and Leuchtenberger, C., "A Rapid Test for Tumor Growth Inhibitors," Ibid., *3*, 401–10 (1943).

(11) Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., and Dische, Z., "Treatment of Spontaneous Breast Cancers in Mice with Pearled Barley," Proc. Soc. Exp. Biol. and Med., *52*, 272–3 (1943).

(12) Laszlo, D., and Leuchtenberger, C., "Inositol, a Tumor Growth Inhibitor," Science, *97*, 515 (1943).

(13) Lewisohn, R., Laszlo, D., Leuchtenberger, C., Leuchtenberger, R., and Dische, Z., "Presence in Various Grains of Factors Inhibiting Tumor Growth," Proc. Soc. Exp. Biol. and Med., *52*, 269–72 (1943).

(14) Leuchtenberger, C., Lewisohn, R., Laszlo, D., and Leuchtenberger, R., "'Folic Acid,' a Tumor Growth Inhibitor," Ibid., *55*, 204–5 (1944).

(15) Lewisohn, R., Leuchtenberger, R., and Leuchtenberger, C., "The Action of Xanthopterin on Tumor Growth," Ibid., *56*, 144–5 (1944).

(15a) Lewisohn, R., Personal Communication (Sept., 1944).

(16) Burk, D., Hesselbach, M. L., MacNeary, D. F., and Winzler, R. J., "The Vitamer Concept," Trans. N. Y. Acad. Sci., (2), *6*, No. 8, 1944.

(17) Am. Assoc. Adv. Sci. Symposium on Cancer (Gibson Island, Md.), 1944. To be published.

(18) Miller, J. K., Miner, D. L., Rusch, H. P., and Baumann, C. A., "Diet and Hepatic Tumor Formation," Cancer Research, *1*, 699–708 (1941).

(19) Burk, D., Kensler, C., Rhoads, C. P., Sugiura, K., and du Vigneaud, D., Reported at 35th Meeting of Am. Assoc. Cancer Research, Boston, 1942.

(20) Wright, L. D., and Welch, A. D., "The Production of Folic Acid by Rat Liver in vitro," Science, *98*, 179–82 (1943).

(21) Editorial Comment, "Vitamin Bc, 'Folic Acid,' and Xanthopterin," Nutritional Reviews, 2, 131–3 (1944).

(22) Wieder, S., "The Present Status of *Lactobacillus casei* Factor" (Brochure), Lederle Laboratories, Inc., June, 1944.

(23) Woglom, W. H., in Report of the Dean of the School of Medicine, Columbia University, for the Academic Year Ending June 30, 1940, p. 29.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 118, 332-339 (1967)

Metabolism of *myo*-Inositol in Animals

II. Complete Catabolism of *myo*-Inositol-^{14}C by Rat Kidney Slices[1]

CHARLES F. HOWARD, JR.[2] AND LAURENS ANDERSON

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisconsin

Received May 16, 1966

myo-Inositol-2-^{14}C is not degraded to respiratory $^{14}CO_2$ by nephrectomized rats. This and the results of earlier tracer experiments suggest that the kidney is the only organ of importance in inositol catabolism. Rat kidney slices could convert at least 4.7 μmoles of *myo*-inositol-2-^{14}C to $^{14}CO_2$/gm tissue/hour at inositol concentrations (in the incubation medium) of 12 mM or greater. This is many times the rate obtainable with homogenates but is probably less than the true catabolic rate of the tissue. Cortex, medulla, and the nephron tubules of cortex gave about equal rates, but that of papilla was negligible. Over the range 0.75–9.5 mM (inositol in the medium) the compound was taken up against a concentration gradient. The data indicate that a rat's kidneys can catabolize all of the inositol ingested in an average daily diet. Glucuronate isolated from kidney slices incubated with *myo*-inositol-2-^{14}C was the D-isomer. Substantially all the radioactivity was in C-5, as found with purified inositol oxygenase by Charalampous [*J. Biol. Chem.* **235**, 1286 (1960)].

The importance of the kidney in the animal catabolism of *myo*-inositol was indicated by the discovery, in rat kidney, of an enzyme or enzymes that cleave inositol to glucuronate (8). Subsequent investigators demonstrated the formation of D-glucuronate and L-gulonate from labeled inositol *in vivo* (3, 5), but it was found that inositol contributed little label to ascorbic acid in the rat (5). To explain this finding, Burns and collaborators suggested that the conversion of inositol to glucuronate and thence to gulonate occurs only in the kidney, which, in the rat, lacks the enzymes for oxidizing gulonate to ascorbate. The fact that inositol is not an effective precursor of ascorbic acid in rats was confirmed in our laboratory (10). We were then led to extend the plausible suggestion of Burns *et al.* in order to rationalize other "anomalous" results obtained (see *Discussion*) when labeled *myo*-inositol was given to rats. Our hypothesis was that the entire process of *myo*-inositol catabolism takes place in the kidney, with little leakage to the bloodstream of intermediates, at least those prior to glucose.

Direct evidence concerning the role of the kidney in inositol catabolism *in vivo* was obtained with nephrectomized rats, which did not form $^{14}CO_2$ from *myo*-inositol-2-^{14}C. Beyond this a direct test of the "organ compartmentation" hypothesis did not appear feasible, but it seemed of interest to establish whether kidney tissue can effectively carry out the complete catabolism of inositol. The oxidation of inositol to CO_2 by rat kidney homogenate preparations was reported by Richardson and Axelrod (25), but the rates were very low. In the work described in this paper we accordingly devoted

[1] Supported by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and by a predoctoral fellowship awarded to C.F.H. by the Division of General Medical Sciences, National Institutes of Health. Paper I of this series: Anderson and Coots (3).

[2] Present address: Oregon Regional Primate Research Center, Beaverton, Oregon.

most of our attention to kidney slices. These were found to be highly active.

MATERIALS AND METHODS

Substrates, reference compounds. *myo*-Inositol-2-^{14}C (sp. act. 0.78 mC/mmole)[3] was synthesized in this laboratory (12), and uniformly labeled *myo*-inositol-^{14}C (0.1 mC/mmole) was kindly provided by Professor S. J. Angyal, University of New South Wales. These were diluted with unlabeled *myo*-inositol to the specific activities required in the various experiments. Glucuronolactone-6-^{14}C (0.29 mC/mmole) was obtained from the National Bureau of Standards. L-Gulonamide was a gift from Professor Frank Loewus.

Homogenates and slices. Rats purchased from the Holtzman Co., Madison, Wisconsin, were maintained on a stock (natural) ration and were used in the weight range of 250–300 gm. They were stunned and decapitated, and the excised kidneys were placed in an ice-cold buffer-salts medium without glucose (26) until used. After the desired number of rats were killed, slices, 0.1–0.2 mm thick, were cut free-hand. Papilla was routinely removed during slicing so that the tissue used consisted only cortex and medulla (see *Results*). The slices were kept in cold buffer-salts medium without substrate, and incubation was always started 1.5 hours after killing the first rat. This served to standardize the preincubation treatment of the slices regardless of whether many or only a few kidneys were used. Tubules of the kidney cortex were prepared by Dr. Claude Arnaud according to the method of Burg and Orloff (4). They were found to be primarily nephron tubules with only slight contamination by collecting tubules. Homogenates were prepared in 0.25 M sucrose with a Potter-Elvehjem homogenizer.

Incubations were carried out in Warburg flasks, with shaking, at 37°. In experiments with slices each flask was charged with 300 mg (wet weight) of tissue and 3 ml of buffer-salts medium containing *myo*-inositol-2-^{14}C at the desired concentration. Oxygen–carbon dioxide (95:5) was used as the gas phase, except where otherwise noted. Two methods were employed for trapping metabolic $^{14}CO_2$. In the flowing gas procedure, the gas passed through the flask and into a Vigreux column containing ethanolamine-Cellosolve (20). In the closed flask procedure, the flasks were gassed and sealed with a rubber injection stopper. If CO_2 evolution was to be measured for a period not including the initial phase of the incubation, the flasks were evacuated, regassed, and resealed at the appropriate time. On completion of the incubation concentrated hydrochloric acid was injected to stop the reaction. This was followed by ethanolamine-Cellosolve, delivered to the center well. Aliquots of the absorbent were pipetted into counting vials and the final solution was made to 0.6 ml ethanolamine, 8 ml methyl Cellosolve, and 10 ml toluene plus scintillators. Radioactivity was determined with a Packard Tri-Carb scintillation counter.

To test whether the slices maintained viability during the experimental period used, the oxygen consumption of several lots (100 mg per flask) was measured. Inositol was added to the medium at levels ranging from 0 to 27.8 mM, but it had no consistent effect. The average consumption during hour 1.5–2.5 (i.e., hour 3–4 after killing the rat) fell less than 10% from the value of 86 ± 7 μmoles of oxygen per gram measured during hour 0.5–1.5.

Degradation of intermediates. Glucuronate was reduced to gulonate according to Charalampous (7), and gulonolactone was converted to gulonamide in ammonia-saturated ethanol. The procedure of Eisenberg (13) was used for periodate degradations. Any carbon dioxide formed was trapped, titrated, and counted. Formaldehyde and formic acid were separated from the reaction mixture, and the formaldehyde was converted to its dimedon derivative. Portions of the solid organic compounds were oxidized to carbon dioxide by the method of Van Slyke *et al.* (28) for determination of their specific activities. Sodium formate was oxidized to carbon dioxide with mercuric chloride (24)

To see whether C-1 and C-2 from gulonamide were segregated cleanly (as glyoxylamide) from C-3, 4, and 5 in the periodate oxidation, a sample of L-gulonamide-1-^{14}C prepared from D-glucuronolactone-6-^{14}C was oxidized. Two to three per cent of the ^{14}C appeared as $^{14}CO_2$, indicating that a similar portion of C-2 was contributing to the middle carbon (formate) fraction.

RESULTS

Failure of nephrectomized rats to catabolize inositol.[4] Before being subjected to nephrectomy the two rats used had received test doses of *myo*-inositol-2-^{14}C on several occasions, and had exhibited normal inositol catabolism. This means that the activity of the respiratory CO_2 rose to a maximum 1–1.5 hours after the injection, and 15–25% of the ^{14}C administered was expired within 4 hours (10, 22, 23). The animals were bi-

[3] We thank Dr. L. V. Hankes of the Brookhaven National Laboratory for checking this value by combustion and counting in a gas-phase proportional counter.

[4] In collaboration with P. A. Weinhold.

laterally nephrectomized under pentobarbital anesthesia, and when they regained consciousness, each was given 0.81 mg of *myo*-inositol-2-^{14}C (undiluted) by intraperitoneal injection. Respiratory carbon dioxide was sampled at intervals for 24 hours and the samples were plated as barium carbonate. None had any detectable radioactivity.

Catabolism in kidney homogenates. Homogenates were studied by the closed flask method, at various dilutions, and with a number of combinations of cofactors and activators. The substrate was inositol-^{14}C, usually uniformly labeled. For purposes of calculation, mμmoles of $^{14}CO_2$ trapped (divided by six for uniformly labeled substrate) was taken as equivalent to mμmoles of inositol catabolized. The highest rate of catabolism, 7.9 mμmoles/gm tissue per hour, was obtained with a substrate concentration of 21 mM and a 75% homogenate (final concentration) fortified with NAD, NADP, ATP, nicotinamide, and magnesium ions, incubated in air. This catabolic rate is not significant in relation to the estimated amount of inositol which must be handled by the rat daily.

Catabolism in kidney slices: Activities of the different anatomical areas. Before detailed studies were carried out with slices, the question was asked whether the ability to catabolize inositol was localized in a particular area or functional unit of the kidney.

TABLE I

CATABOLISM OF *myo*-INOSITOL BY DIFFERENT PORTIONS OF RAT KIDNEY

Area	Wet wt. slices (mg)	Incub. time (hours)	Inositol conc. (μmoles/ml)	Inositol-2-$^{14}C \rightarrow {}^{14}CO_2$ (μmoles/gm tissue)[b]
Cortex[a]	210 ± 5	1.5	5.64	1.92 ± 0.12
Medulla[a]	228 ± 10	1.5	5.64	1.83 ± 0.43
Papilla[a]	138 ± 5	1.5	5.64	0.01
Tubules	75[c]	1	4.58	1.45
	75[c]	2	4.58	2.44

[a] Two separate experiments with a total of four flasks of cortex, four of medulla, and two of papilla.

[b] From zero to time indicated, by the closed flask method.

[c] Wet weight estimated from mg N.

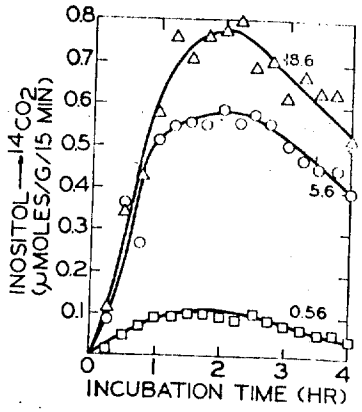


FIG. 1. Time course of the catabolism of inositol-2-^{14}C to $^{14}CO_2$ by kidney slices. The figures at the right are the initial concentration of inositol (μmoles/ml) in the medium. The flow gas method of CO_2 collection was used.

This seemed possible in view of the considerable physiological and anatomical differentiation of that organ. Also suggestive was the observed narrow localization of the kidney necrotic effect of the inositol antagonist 2-*O*,*C*-methylene-*myo*-inositol (29).

Table I shows the results obtained by incubating slices from the three principal zones of the kidney, and isolated tubules. Neither the substrate concentrations nor the incubation regime conformed to the optimum worked out in later experiments, but it is clear that cortex, medulla, and isolated tubules are about equally active in catabolizing inositol to CO_2. Papilla has very little activity, which is the basis for removing it before slicing in subsequent experiments.

Time course of $^{14}CO_2$ formation from inositol-2-^{14}C. When slices were incubated by the standard procedure with several different concentrations of inositol, the curves shown in Fig. 1 were obtained. It may be seen that the evolution of radioactive carbon dioxide was maximal between 1.5 and 2.5 hours regardless of the substrate concentration used. A fourth run with 0.046 mM inositol/ml, when plotted on an expanded vertical scale, gave a curve similar to those shown.

If incubation of slices was started within 12 minutes after killing the rat, a somewhat differently shaped curve resulted. The

production did not attain a maximal rate until 2 2.5 hours and then declined only lightly to the end of the 4 hour collection period. The maximal rate of $^{14}CO_2$ evolution at an inositol concentration of 5.6 μmoles/ml was about two thirds of that observed with the standard procedure.

Variation of catabolic rate with inositol concentration. In view of the foregoing results, the rate of $^{14}CO_2$ evolution during hour 1.5 2.5 of the standard incubation (i.e., hour 3 to 4 after killing the first animal) was taken as the best measure of inositol catabolism in kidney slices. Slices were incubated with various concentrations of inositol, and the $^{14}CO_2$ output during the period of maximal evolution was measured by both the closed flask and flowing gas methods. The results are presented in Fig. 2. Higher values were obtained by the flowing gas method than by the closed flask method, but in both cases the rates of $^{14}CO_2$ production at first increased and then leveled off at a substrate concentration of 8–12 mM. Averaging all of the values obtained at inositol concentrations of 12 mM and greater gave, as figures for the maximal rate of $^{14}CO_2$ production under the conditions used, 4.7 μmoles/gm tissue/hour under flowing gas and 3.1 moles/gm tissue/hour in closed flasks.

Inositol flux during incubation of kidney slices. To better understand how the catabolic rates observed in the previous experiments may be related to physiological rates, it was necessary to determine the effect of increasing concentrations of exogenous inositol on the endogenous levels. Kidney slices held in cold buffer-salts in the usual way were accordingly incubated for 1 hour with increasing concentrations of unlabeled inositol and then separated from the medium by filtration and frozen. The free inositol content of the slices was determined by homogenizing them in water and assaying with *Saccharomyces carlsbergensis* (1). Assay of an aliquot following overnight autoclaving in 6 N H_2SO_4 and then neutralization (16) gave the total inositol. The difference between the total and free inositol represents bound inositol. Intracellular free inositol concentrations were computed from the values for free inositol in the slices by correcting for the extracellular fluid volume of the tissue, taken to be 25% (27).

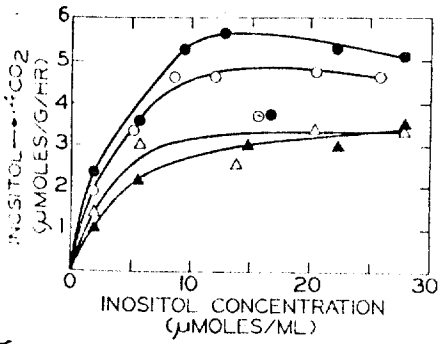


FIG. 2. Effect of inositol concentration on metabolism to carbon dioxide. The top two curves obtained by the flowing gas method and the two by the closed flask method.

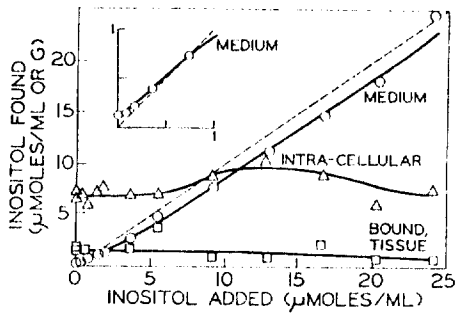


FIG. 3. Effect of varying inositol concentrations on the distribution of free and bound inositol in kidney slices and in the medium. Flasks were gassed with oxygen-carbon dioxide, closed, incubated for 1 hour, and assayed. Each point represents 2–3 separate experiments at inositol concentrations of 9 μmoles/ml or below and one experiment at higher concentrations. Values for the medium would have fallen on the dashed line if no changes had occurred during incubation.

The data, plotted in Fig. 3, show that at *final* external concentrations between 0.75 and 9.5 mM (initial, 0.75–11 mM) there was uptake of inositol against a gradient. At the upper end of this range an intracellular free inositol concentration of 9.5 mM was attained. This value was not raised by further increasing the external inositol; in fact, at very high external inositol levels the intracellular concentration declined somewhat. At external concentrations below 0.75 mM the inositol in the *medium* appeared to increase slightly.

Isolation and degradation of D-*glucuronate-5-*^{14}C *from kidney slices.* Slices, 2.01 gm from 6 rats, were distributed among four flasks and incubated 1.25 hours with *myo*-inositol-2-^{14}C (about 3.1 μmoles/ml). The flask contents plus rinse water were homogenized and the protein was precipitated with perchloric acid. The supernatant fluid was brought to pH 8.5 with potassium hydroxide, thus removing the perchlorate and assuring the opening of lactone rings. Adsorption on Dowex 1-formate and elution with 0.7 N formic acid gave 65 μeq, by titration, of anionic material. This was subjected to gradient elution and resolved into several radioactive peaks as shown in Fig. 4.

From the experience of Loewus and Kelley (21) it was supposed that peak 4 would be gulonate, and peak 5, glucuronate. Paper chromatography of a lactonized aliquot of peak 5 showed that the radioactivity moved with authentic glucuronolactone. The radioactive substance of peak 4, however, failed to co-crystallize with authentic L-gulonolactone. No further attempts were made to identify it or the substances of peaks 1–3.

Carrier D-glucuronic acid, 191.6 mg, was added to the peak 5 solution, the pH was adjusted to 7.4, and sodium glucuronate was crystallized by ethanol addition. The recovered crystals (200 mg, 96% yield) had a specific activity of 121 dpm/μmole, unchanged by recrystallization. Reduction gave gulonolactone, m.p. 184.5–186°, of increased specific activity (135 dpm/μ... showing that the glucuronate rece... by the carrier precipitation was the D-i... If labeled L-glucuronate had been pre... the carrier precipitate it would have ... eliminated, with a consequent lowerin... the specific activity of the carrier-dil... product, when the latter, after reduc... was crystallized as gulonolactone (5).

Periodate degradation of 0.095 mmo... sodium gulonate from the lactone (di... with carrier to 66 dpm/μmole) yielded ... following results: carbon dioxide from ... (C-6 of glucuronate), inactive; formal... hyde from C-6 (C-1 of glucuronate), ... tive; sodium formate from C-2–5 (C-5 ... of the glucuronate), 20 dpm/μmole. The latter figure is equivalent to 80 dpm/μmole of gulonate and fully accounts for the radioactivity.

Periodate degradation of 0.046 mmole ... gulonamide, m.p. 122.5–124° (Reference ... prepared from the undiluted lactone ... sodium formate which should be de... from three carbons of the gulonate ch... but should not include C-2. The spe... activity was 5 dpm/μmole, or 15 dpm/... of gulonamide (11% of the total). By d... ence, 89% of the activity of the gulon... must be in C-2 (C-5 of glucuronate). ... true figure is somewhat higher since ... shown in a control degradation (*Me*... a small percentage of C-2 "leaks" into ... formate fraction.

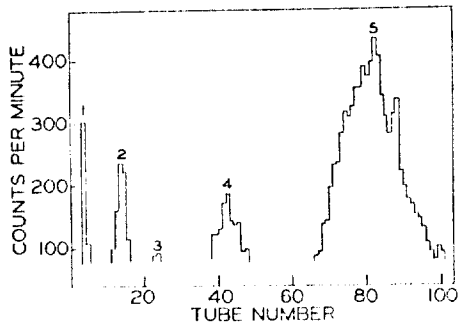


FIG. 4. Elutogram of the anionic material obtained from rat kidney slices following incubation with *myo*-inositol 2-^{14}C. Twelve ml of Dowex 1 (X8, 200–400 mesh, formate form) were slurried into a 1 × 30-cm glass column. Elution was with formic acid, 0 → 0.06 N (Reference 21). Flow rate was about 1 ml/minute, and 5 ml fractions were collected.

DISCUSSION

It is evident, from the results re... above, that rat kidney tissue is able to ... out a vigorous catabolism of *myo*-in... Kidney slices incubated in a simple ... salts medium and supplied with *myo*-... as the only organic substrate were ... sustain this catabolism for several ... The fact that the rate of $^{14}CO_2$ evoluti... slow at the beginning of the incubati... increased for a period of an hour or ... of course, an artifact of the experi... situation. This initial period was required ... for the process of inositol uptake ... kidney cells to establish a steady state ... ing of the pool of inositol which was ... oxidized.

That the uptake of inositol by kidn... would be an active process was to be a

pected from the observations of Dawson and Feinkel (11), who found the inositol levels in many organs, including kidney, to be higher than that in blood in intact animals. Our results confirm this expectation, and show that the process operated over a considerable range of concentration differences. Further information is provided by the recent data of Hauser (17); using radioactivity as an indicator this investigator demonstrated active uptake of inositol by rat kidney slices at the rather low external concentration of 0.017 mM. Since our data for the range 0–0.75 mM, and Hauser's for 0.017 mM, indicate a small net flux of inositol from the tissue to the medium, an exchange process may be involved; or the figures may represent a unidirectional uptake by undamaged cells that is more than balanced, at these concentrations, by loss from damaged cells.

Even if the intact cells exchange inositol with the medium, the intracellular inositol may not have become completely labeled in our experiments, particularly at the lower external concentrations. Hauser found that the specific activity of intracellular free inositol was still much less than the extracellular after a 2-hour incubation. On the other hand it is possible that the pool of inositol which is undergoing catabolism is only a portion of the total intracellular free inositol. In this case the degree of labeling of the active pool might approach that of the medium after 1–1.5 hours of incubation.

It is interesting that at high external inositol levels the intracellular inositol concentration reached a limit. Moreover, the external inositol concentration just sufficient to raise intracellular inositol to the maximum was the same as that which just gave maximal catabolic rate. This result suggests that the transport system becomes saturated before the catabolizing system.

The reason for the much lower metabolic activity of homogenates, as compared with slices, was not discovered in our limited work with the former. In view of the high K_m of inositol oxygenase *in vitro* (6) very high substrate concentrations may have to be used to get maximal rates with homogenates. The quick "reaction inactivation" of inositol oxygenase would also contribute to the low activity of these preparations. In any case it is clear that all rates of inositol oxidation so far obtained with kidney homogenates represent only a small fraction of the capability of the whole tissue. It would therefore appear unwarranted to conclude from observations with homogenates, as some authors have, that there are two pathways for the initial stages of inositol catabolism in the kidney.

In order to use our data to arrive at an estimate of the capacity of the rat kidney for the complete catabolism of inositol, some assumptions are required. The first of these, as mentioned in the Results section, is that the release of $^{14}CO_2$ from inositol-^{14}C involves complete oxidation of the compound. This will hold for *myo*-inositol-2-^{14}C if catabolism is by the glucuronate-xylulose pathway (see below) since in this scheme only the 1-carbon of inositol is lost in an early decarboxylation. The remaining carbons are converted to hexose phosphate or triose phosphate, and the eventual release of $^{14}CO_2$ from these through the operations of the Krebs cycle may be defined as complete oxidation. A second, reasonable assumption is that rates of oxidation in the intact kidney under physiological conditions are at least as great as those achieved, at similar *intracellular* concentrations, in slices incubated *in vitro*. A factor which will tend to make our calculated rates less than the true rates is the probability, discussed above, that the intracellular inositol did not become fully labeled.

If a 300-gm rat consumed 20-gm per day of a diet containing 3 mg of inositol per gram,[5] and if all of this inositol were absorbed, then the daily load, ignoring biosynthesis in the rat's own tissues, would be 333 μmoles. If the weight of the kidneys was 3 gm, and they operated at the rate of 4.7 μmoles/gm/hour (the maximum calculated from our $^{14}CO_2$ data), their capacity would be 338 μmoles/day. Since analyses (15; unpublished data obtained in this laboratory[6]) show that the normal intracellular free inositol concentration in rat kidney is prob-

[5] A typical value for rat rations compounded of natural feeds (analyses in the authors' laboratory).

[6] Our value is 8 μmoles/gm cell contents, assuming all the inositol is intracellular. Eisenberg and Bolden's figure is somewhat higher.

ably close to the maximum found under our conditions, the tissue should easily achieve the same rate *in vivo*. The quoted rate is itself a conservative estimate, hence it seems safe to conclude that the kidneys of a rat can dispose of the usual load of ingested inositol, plus additional inositol biosynthesized in the tissues. Thus if, on other grounds, one arrives at the hypothesis that inositol catabolism in the rat is uniquely a function of the kidneys, the present data indicate that the kidneys have the capacity for this function.

The experiment with nephrectomized rats strongly implicates the kidney as the organ in which inositol catabolism is initiated. Although a minimal number of rats was involved, the animals served as their own controls, and the results were clear-cut. The fact that these animals did not convert inositol-^{14}C to $^{14}CO_2$ shows they were not cleaving inositol to D-glucuronate, since nephrectomized rats are known to be able to catabolize D-glucuronate (14). If organs other than the kidney have the ability to cleave inositol, this ability was not manifest under the circumstances prevailing. The extrarenal cleavage of inositol in *intact* rats is not of course ruled out by this experiment, but the evidence cited next suggests that the role of other organs in normal inositol catabolism is probably minimal.

The results of many *in vivo* tracer experiments have led to the conclusion that the effective pathway of inositol catabolism is via glucuronate, the xyluloses, and the pentose phosphate cycle (18). However, as stated in the introduction, there are some inconsistencies in the tracer data. These inconsistencies are largely explained by the hypothesis of "organ compartmentation" (completion in the kidney). In addition to clarifying the observation that inositol is not a good precursor of ascorbic acid, this hypothesis explains why the maximum in the $^{14}CO_2$ expiration curve following a dose of inositol-^{14}C occurs some hours before liver and muscle glycogen are appreciably labeled (see 2, 3, 10; further, unpublished data has been obtained in our laboratory). Apparently, glucose formed from inositol in the kidney is released only slowly. The fact that the glucose units of liver and muscle glycogen derived from *myo*-inositol 2-^{14}C are nearly equally labeled in positions 1 and 6 (Reference 3), instead of largely in 6 as predicted from the glucuronate-xylulose pathway, may be attributed to secondary processes occurring during the long residence of the glucose in the kidney. When phlorizinized rats are given *myo*-inositol 2-^{14}C, the urinary glucose, which is presumably formed in shorter time in the kidney, has considerably higher activity in C-6 than in C-1, as expected (P. A. Weinhold, unpublished data, 9). The labeling in C-1 is still substantial, however, and the mechanism by which this is achieved remains to be elucidated.

The isolation of labeled glucuronate from kidney slices incubated with *myo*-inositol-^{14}C provides additional support for the conclusion that animals catabolize inositol via the glucuronate-xylulose pathway. The fact that the label was in position 5 shows that glucuronate was derived directly from the inositol, for cycling of the label through glucose would put it in positions 1 and 6. Presumably, the inositol oxygenase of Charalampous is responsible for the formation of D-glucuronate *in vivo*, since it was shown (7) that the two variants of this enzyme cleave 2-labeled inositol to 5-labeled D- and L-glucuronate, respectively.

In previous examinations of urinary glucuronate derived from labeled inositol only the D-isomer has been definitely detected (5, 10). Although isolation from slices should afford a better opportunity of detecting L-glucuronate, if it is indeed formed in the tissue, our data likewise suggest that it is not present. Before its absence is concluded, however, a more definitive experiment should be performed in which L-glucuronate is used as carrier.

REFERENCES

1. Atkin, L., Schultz, A. S., Williams, W. and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.* **15**, 141 (1943).
2. Anderson, L., and Coots, R. H., *Fed. Proc.* **17**, 182 (1958).
3. Anderson, L., and Coots, R. H., *Biochim. Biophys. Acta* **28**, 666 (1958).
4. Berg, M. B., and Orloff, J., *Am. J. Physiol.* **203**, 327 (1962).
5. Burns, J. J., Troupin, N., Evans, C. [illegible]

DOPOULOUS, N., AND AGRANOFF, B. W., *Biochim. Biophys. Acta* **33,** 215 (1959).
6. CHARALAMPOUS, F. C., *J. Biol. Chem.* **234,** 220 (1959).
7. CHARALAMPOUS, F. C., *J. Biol. Chem.* **235,** 1286 (1960).
8. CHARALAMPOUS, F. C., AND LYRAS, C., *J. Biol. Chem.* **228,** 1 (1957).
9. CHARGOLLAIS, E., AND POSTERNAK, T., *Helv. Chim. Acta* **48,** 280 (1965).
10. COOTS, R. H., Ph.D. Thesis (with L. Anderson), University of Wisconsin (1958); *Dissertation Abstr.* **19,** 1541 (1959).
11. DAWSON, R. M. C., AND FREINKEL, N., *Biochem. J.* **78,** 606 (1961).
12. DRUMMOND, G. I., ARONSON, J. N., AND ANDERSON, L., *J. Org. Chem.* **26,** 1601 (1961).
13. EISENBERG, F., JR., *J. Am. Chem. Soc.* **76,** 5152 (1954).
14. EISENBERG, F., JR., *Federation Proc.* **16,** 176 (1957).
15. EISENBERG, F., JR., AND BOLDEN, A. H., *Nature* **202,** 599 (1964).
16. HALLIDAY, JUNE W., AND ANDERSON, L., *J. Biol. Chem.* **217,** 797 (1955).
17. HAUSER, G., *Biochem. Biophys. Res. Commun.* **19,** 696 (1965).
18. HOLLMANN, S., AND TOUSTER, O., "Non-Glycolytic Pathways of Metabolism of Glucose," pp. 90–107. Academic Press, New York (1961).
19. HUDSON, C. S., AND KOMATSU, S., *J. Am. Chem. Soc.* **41,** 1141 (1919).
20. JEFFAY, H., AND ALVAREZ, J., *Anal. Chem.* **33,** 612 (1961).
21. LOEWUS, F. A., AND KELLY, S., *Arch. Biochem. Biophys.* **102,** 96 (1963).
22. MOSCATELLI, E. A., AND LARNER, J., *Arch. Biochem. Biophys.* **80,** 26 (1959).
23. NIXON, D. A., *Nature* **187,** 77 (1960).
24. PIRIE, N. W., *Biochem. J.* **40,** 100 (1946).
25. RICHARDSON, K. E., AND AXELROD, B., *Biochim. Biophys. Acta* **32,** 265 (1959).
26. ROBINSON, J. R., *Biochem. J.* **45,** 68 (1949).
27. ROSENBERG, L. E., BLAIR, ALBERTA, AND SEGAL, S., *Biochim. Biophys. Acta* **54,** 479 (1961).
28. VAN SLYKE, D. D., PLAZIN, J., AND WEISIGER, J. R., *J. Biol. Chem.* **191,** 299 (1951).
29. WEINHOLD, P. A., IYER, N. T., AND ANDERSON, L., *Proc. Soc. Exptl. Biol. Med.* **112,** 105 (1963).

THE JOURNAL OF VITAMINOLOGY 12, 1–9 (1966)

STUDIES ON MYOINOSITOL
II. POSSIBLE ROLE OF DIETARY MYOINOSITOL ON THE GROWTH PROMOTION OF YOUNG RATS

AKIRA KOTAKI, KAZUKO NATSUME, YOSHIKO YAMAMOTO AND KUNIO YAGI[1]

Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya

(Received September 13, 1965)

Although the nutritional need of myoinositol has been widely observed at the cellular level of organisms (1–5), its vital essentiality in intact mammals is scarcely accepted since the demonstration of any symptoms caused by myoinositol deficiency is rather said to be extremely difficult probably because of its possible biogenesis in some mammalian tissues (6–9). However, in spite of this unequivocal fact, the results described in the preceding paper (10) has revealed the beneficial effect of dietary myoinositol on the growth of young rats. Hence, it may be necessary to indicate the reasons why myoinositol is capable of promoting the growth of young animals.

Concerning this problem, Agranoff and Fox (11) has suggested an antagonistic action between choline and myoinositol during the rapid synthesis of cells in developing animals. The main subject of this study is to accomplish some experimental approaches to their assumption, in order to clarify the nutritional role of myoinositol on the growth of young animals. In the present paper will be described the growth suppression of young rats accompanied by a rise of P/N ratio of their hepatic lipid fraction, which is provoked by the successive administration of a large excess myoinositol. In addition will be described the restoration of retarded growth as well as elevated P/N ratio by feeding the rats on a high protein diet or with increasing amounts of choline.

EXPERIMENTAL

1. Animals and Ration

In breeding experiments, Wistar strain of 20 day old male rats (average body weight, 50 g) were used, while 2 month old ones (average body weight, 120 g) in the control experiments. Unless otherwise mentioned, the basal diet and vitamin mixture were prepared according to the preceding paper (10). In one experiment, casein content of the basal diet was elevated from 22 to 30 per cent, whereas sucrose content was decreased from 64.5 to 56.5 per cent. Animals were housed in the individual cage, and grown in the room maintained at 18–23°.

[1] 小滝 晃, 夏目和子, 山本良子, 八木国夫.

2. Administration of Myoinositol and Choline

Rats were divided into 5 groups, and the 4 groups were administered compulsorily with 0.5 ml of myoinositol solutions at a definite time every day as shown in Table 1. Each group consisted of 3 to 8 animals. To minimize the influence

TABLE 1
Administration of Myoinositol and Choline

Experimental group	Addition	
	Myoinositol	Choline
	mg/day	*mg/day*
	Exp. 1	
A	—	1.25
B	0.5	1.25
C	5	1.25
D	10	1.25
E[a]	50	1.25
	Exp. 2	
F	—	1.25
G	0.5	1.25
H	5	10
I	10	20
J[a]	50	100

[a] Administered as suspension.

of the administration of such a large volume of inositol solution, control group (group A) was also administered compulsorily with 0.5 ml of distilled water. In the latter experiment shown in Table 1, a large amount of choline chloride was a large amount of administered simultaneously with myoinositol by dissolving its necessary amounts into each myoinositol solution.

3. Extraction of Hepatic Total Lipids

Extraction of total lipids from liver homogenates was performed according to essentially the same procedure as suggested by Entenman (12). Namely, rats were sacrificed by decapitation after ether anesthesia and the livers were removed and weighed. At this time, bloods were collected from carotid artery in order to examine the level of serum cholesterol. Liver slices were homogenized with five volumes of ethanol-diethyl ether (3 : 1, v/v) by using ultra-turrax. Dry weight of each liver was measured by weighing an aliquot of the homogenate after drying in the oven at 110° for 24 hours. Each homogenate was extracted with 50 ml of the solvent at 60° for an hour, and centrifuged. This extraction procedure was repeated three times. Combined extracts were evaporated to dryness *in vacuo* and the residue was redissolved in 20 ml of petroleum ether (bp 40–60°). The resulting solution was washed twice with each 10 ml of distilled water and was made to definite volume using a volumetric flask.

4. Assay of Lipids

To estimate the total hepatic lipids, an aliquot of petroleum ether extracts was evaporated and the residue was weighed. Phosphorus content in the liver lip-

was determined according to the method of Nakamura (13), after ashing with 60 per cent perchloric acid according to the method of Allen (14). Nitrogen content was determined by a micro-Kjeldahl technique, using the spectrophotometry of Labochinsky (15). Determination of total cholesterol in the liver lipids and serum was performed by the method of Zlatkis (16).

5. Determination of Urinary Myoinositol

Twenty-four hour urine was collected for the assay of myoinositol by housing 3 rats in a cage, the bottom of which was connected to a beaker through a funnel. Myoinositol excreted in urine was estimated by the chemical method using periodate oxidation technique (17, 18), after the removal of inhibitory substances such as ionic substances and sugars by the routine procedure. No particular treatment was made for the removal of urinary glycerol.

RESULTS

1. Inhibitory Effect of Large Excess Myoinositol on the Growth of Young Rats

When young albino rats were fed on a diet described by Forker and Morgan (19), the administration of 0.5 to 2 mg myoinositol apparently stimulated their growth (10). However, as indicated in Fig. 1, its large excess administration does

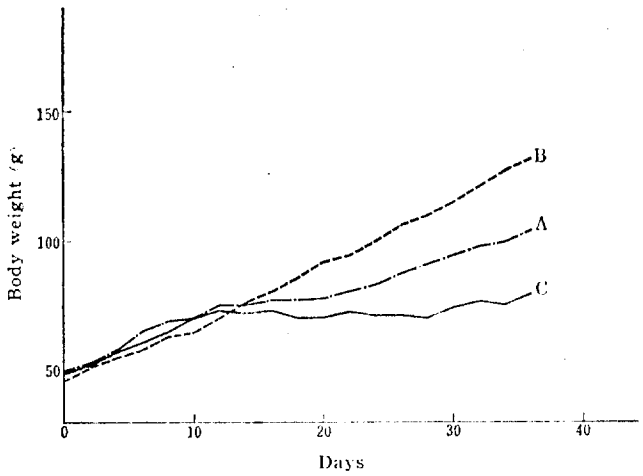


FIG. 1 *Growth Repressing Effect of Myoinositol when Administered in a Large Excess Amount*

A, no addition; B, 0.5 mg/day; C, 50 mg/day of myoinositol.

not always accerelate, but rather appears to repress their growth. When 50 mg or more myoinositol was administered to the young rats daily, retardation of body weight gain was initiated after breeding for about ten days as if rats were fallen into a sort of slight chronic poisoning. Results were fairly reproducible in the three trials of examination. As shown in Table 2, there were no significant differences among these experimental groups in the urinary excretion of myoinositol.

TABLE 2
Urinary Excretion of Myoinositol

Amount of myoinositol administered	Amount of myoinositol in 24 hour urine
mg/day	*mg*
—	0.182
0.5	0.155
5	0.256
10	0.140
50	0.176

[a] Average value of three animals.

Therefore, it may be possible to consider that most part of dietary myoinositol absorbed in body, could be metabolized through the various routes (20).

Relating to the above findings, the effect of the successive administration of large excess myoinositol on the two month old rats were examined as a control run, but no effects were observed during the two months of breeding even in the case of the group administered with more than 0.5 g of myoinositol every day.

Hence, the results may suggest that such growth repressing effects may occur preferentially upon younger rats rather than upon older ones. It might be possible to suppose a difference in the behavior of dietary myoinositol in the body between young and old rats. A reason for such a difference should be attributed to the difference in the rate of the cellular multiplication.

2. *Effect of the Increase of Myoinositol Administration on the Hepatic Lipids*

Since the lipotropic character of myoinositol has been widely accepted, it might be natural to deduce that such an inhibitory action of excess myoinositol on the growth of young animals is, at least partly, due to its influence on the lipid metabolism. From this standpoint, the amount of serum and liver total cholesterol were firstly examined. However, as indicated in Table 3, the elevation of the amount

TABLE 3
Effect of Dietary Myoinositol upon Amount of Serum and Liver Total Cholesterol

Administration of myoinositol	Serum total cholesterol[a]	Liver total cholesterol[b]
mg/day	*mg/ml*	*mg*
—	1.46	14.7
0.5	1.50	13.4
5	1.44	14.5
10	1.41	13.4
50	1.51	15.2

[a] Average value of 18 animals.
[b] mg/g of total hepatic lipids.

myoinositol dosage could not render a significant influence on the concentration of total cholesterol not only in serum, but also in the liver. However, it was found that P/N ratio of hepatic total lipids tends to rise in all the three trials examined by increasing the amount of myoinositol administration, as shown in Table 4

TABLE 4

Effect of Dietary Myoinositol on the P/N Ratio of Hepatic Lipid Fraction

Administration of myoinositol	Series 1			Series 2			Series 3		
	P[a]	N[a]	P/N[b]	P[a]	N[a]	P/N[b]	P[a]	N[a]	P/N[b]
mg/day	*mg*	*mg*		*mg*	*mg*		*mg*	*mg*	
–	18.8	16.6	0.508(±0.123)	22.4	15.4	0.648(±0.099)	20.0	18.8	0.477(±0.060)
0.5	17.3	14.8	0.547(±0.176)	23.4	15.8	0.656(±0.085)	22.3	20.0	0.490(±0.067)
5	22.0	14.9	0.617(±0.118)	22.8	15.4	0.665(±0.101)	22.5	19.7	0.508(±0.132)
10	19.6	13.9	0.666(±0.184)	28.7	17.8	0.770(±0.176)	26.5	21.3	0.543(±0.014)
50	20.0	14.0	0.617(±0.079)	26.4	15.2	0.761(±0.139)	26.6	20.7	0.564(±0.033)

[a] Phosphorus and nitrogen content were calculated as mg/g of total liver lipids.

[b] P/N represents molar ratio between phosphorus and nitrogen. Figures in parenthesis represent the standard deviation. All figures are the average values of 3-8 animals.

the presumption that phosphorous content of the lipid fraction reflects to some extent the amount of total phospholipids while the nitrogen content the amount of nitrogen-containing phospholipids is true, the present result may indicate that the hepatic total phospholipids were increased by the increase of myoinositol administration, whereas those containing nitrogen were relatively decreased. Further support of such interpretation should be desired.

Although there is no direct evidence that this rise of P/N ratio of liver lipids is the very reason for the growth suppressing effect of the excess dietary myoinositol, the fact may strongly suggest the possibility that one of the significant action of dietary myoinositol in the young animal body is, as far as it is given in an appropriate amount, to accerelate the biosynthesis of the phosphatide, that is, extremely important like other phospholipids to produce cellular constructions.

3. Restoring Action of High Protein Diet or Increasing Amount of Choline Administration

As described above, if the successive administration of excess myoinositol provokes a rise of P/N ratio of the hepatic lipids, it should be valuable to take into account the antagonistic action of choline and myoinositol as suggested by Agranoff and Fox (11). Thus, when the nutritional balance is taken into account, it may be necessary to examine the influence of the protein content in the ration or the effect of the simultaneous increase of choline dosage.

Fig. 2 shows the average body weight gain of each group fed on a diet containing 30% casein. It should be noticed that no particular repression of the growth was observed even upon the rats administered with 50 mg of myoinositol daily. In addition, it might be noteworthy that the effective amount of myoinositol for the growth promotion of young rats is elevated a little when they were fed on such a high protein diet. It is likely that the nutritional effect of myoinositol is fairly affected with the amount of proteinous nitrogen taken by the young animals.

As expected from the working hypothesis of this study, the rise of P/N ratio of the hepatic lipids was obviously restrained by feeding the rats on the high protein diet containing 30% casein as shown in Table 5. This fact may be explained by the same reasons as described in the following experiments performed

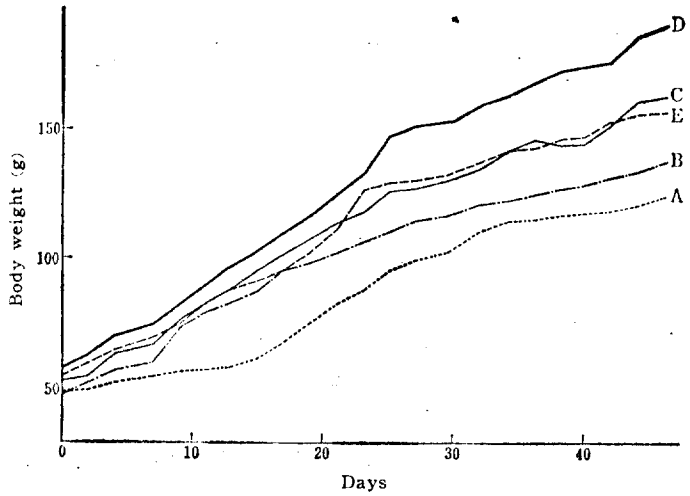


FIG. 2 *The Average Increase in Body Weight of Each Group Fed on a Diet Containing 30% Casein*

A, no administration; B, 0.5 mg/day; C, 5 mg/day; D, 10 mg/day; E, 50 mg/day of myoinositol.

TABLE 5

Analytical Data of Hepatic Lipid Fraction of Rats Fed on a Diet Containing 30% Casein

Administration of myoinositol	Total lipids[a]	Total cholesterol[a]	Phosphorous[b]	Nitrogen[b]	P/N[c]
mg/day	*mg/g*	*mg/g*	*mg/g*	*mg/g*	
—	172	12.2	19.7	17.0	0.517(±0.105)
0.5	189	12.6	19.2	18.0	0.489(±0.036)
5	174	12.9	18.8	15.2	0.553(±0.038)
10	215	14.2	16.2	16.8	0.440(±0.035)
50	162	9.7	15.4	16.3	0.520(±0.014)

[a] mg/g of dry weight of liver.
[b] mg/g of total hepatic lipids.
[c] Molar ratio; figures in parenthesis represent the standard deviation.

by increasing the amount of choline administration. By elevating the protein content, a little repression of the relative amount of hepatic cholesterol was observed although at this time we have no direct informations to explain such a phenomenon from the nutritional standpoint.

If the increase of the dietary nitrogen may indirectly restore the formation of nitrogen-containing phospholipids and, as a result, may control the rise of P/N ratio of the liver lipids, simultaneous increase of choline administration has to be expected to provide similar influence. Actually as indicated in Table 6, repression of the rise of P/N ratio was again observed. This may be evidence for the presence antagonism between choline and myoinositol, and may reveal a flank of nutritional significance of myoinositol. However, the result shown in Table 7 could not demonstrate any particular effects on the body weight gain of young rats by the

TABLE 6

Analytical Data of Hepatic Lipid Fraction of Rats Administered with Increasing Amounts of Choline

Experimental group	Total lipids[a]	Total cholesterol[a]	Phosphorous[b]	Nitrogen[b]	P/N[c]
	mg/g	*mg/g*	*mg/g*	*mg/g*	
F	224	18.9	20.3	18.2	0.503(±0.082)
G	212	17.3	17.3	16.5	0.519(±0.078)
H	265	15.7	15.7	12.9	0.522(±0.096)
I	214	21.0	21.0	19.4	0.498(±0.090)
J	188	26.3	26.3	21.7	0.543(±0.078)

[a] mg/g of liver dry weight.
[b] mg/g of total hepatic lipids.
[c] Molar ratio; figures in parenthesis represent the standard deviation.

TABLE 7

Average Body Weight Gain of Rats Receiving Increasing Amounts of Choline

Experimental group[a]	Average body weight gain
	g/day
F	1.9
G	2.5
H	2.1
I	2.4
J	1.1

[a] See Table 1. Figures are the average values of 5 trials.

simultaneous increase of both choline and inositol. In addition, the rats administered with 100 mg of choline chloride per day hardly showed an increase in body weight. This might be partly due, because of the bitter taste of choline different from myoinositol, to the technical difficulty of the compulsory administration of choline chloride orally to young animals daily in larger amounts.

DISCUSSION

The vitamin action of myoinositol in the body of higher animals is generally considered to be equivocal chiefly because of its biogenesis in their tissues. Actually, however, its growth promoting action was demonstrable upon young rats, although it was almost impossible to show any symptoms caused by its deficiency (10). It has been hoped to elucidate the true mechanism of the action of myoinositol upon growth of young animals. The results mentioned above would indicate, at least in part, a clue to explain the mechanism.

As well known, myoinositol is widely distributed in the mammalian body in the form of phosphatide of which biological function is recently proved to be very significable (21–24). Hence, the idea that dietary myoinositol possesses some beneficial effects on the phosphatide synthesis would be naturally accepted, even if the major part of the supply of myoinositol is endogenous.

Moreover, it should be considered that every biological function involved in one cell is thought to be compartmentized by membranes through which the

intermovement of substances was controlled. Therefore, membrane is an important structural stuff for the maintenance of the functional cell constructions. It is Hanahan's suggestion (25) that phospholipid content of a tissue would merely be a reflection of its membrane expance. From these considerations, it is natural to suppose that the rate of phospholipid biosynthesis is fairly proportional to that of at least cell multiplication. The growth delay observed in the myoinositol deficient rats (10) would be, at least partly, due to the repression of its phosphatide biosynthesis.

After these considerations, it is not difficult to suppose that myoinositol has in animal body has an important role in phosphlipid biosynthesis. Actually, a possible intervention of myoinositol into the phospholipid biosynthesis was demonstrated by the present results: the large excess administration of myoinositol has an inhibitory effect on the growth of young rats, which was capable of being recovered to some extent by elevating the protein content in the basal ration and probably by increasing the dosage of choline. The fact should be referred to the findings of Agranoff and Fox (11) that dietary myoinositol is rather inhibitory for the growth of chicks when it was given to those fed on a choline deficient diet. Similar findings were reported by Handler (26) and Jukes (27). It seems likely that antagonistic action between choline and myoinositol may be rather a necessary phenomenon in the developing animals.

As noted above, excess administration of myoinositol to the developing rats provoked the rise of P/N ratio of the liver lipids, but the simultaneous increase of the amount of casein or choline could repress the rise of this ratio. This fact may give further support on the view of antagonism between choline and myoinositol through a possible influence upon the phospholipid metabolism. Recognizing the conclusion, similar result could be expected in the case of choline deficiency too. Hence, as far as these two factors are supposed to have influence on each other on biosynthesis of their phosphatides, nutritional significance of dietary myoinositol has to be always taken into account in connection with the nutritional action of dietary choline. When these two factors are administered in a proper amount, beneficial effect on the growth of young animals would be expected.

Thus, the present result that the administration of a moderate amount of myoinositol promotes the growth of young animals could be interpreted as follows: (*a*) exogenous myoinositol could be the precursor of the inositol phosphatide, which is an essential component for cell division, (*b*) excess dosage of myoinositol disturbs the phospholipid metabolism, *e.g.* inhibits the synthesis of choline phosphatide which indicates an intervetion of myoinositol into phospholipid metabolism. Therefore, the increase of the amount of myoinositol in the diet can promote the growth of the animals so far as its amount is not enough to disturb the phospholipid metabolism.

Concerning the utilization of the exogenous myoinositol, the reports of Margolis and Heller (28) and Petzold and Agranoff (29) may be referred. The former researchers observed in their study using tritium-labeled myoinositol, that the relative incorporation of radioactivity into brain phospholipid fraction is more striking in developing animals than in older ones (28). This report concludes the utility of exogenous myoinositol for young animals. On the other hand, Petzold *et al.* (29) found that the incorporation of tritium-labeled cytidine nucleotide by liver

extracts into CDP-diglyceride which is a possible precursor for many phospholipids, is considerably inhibited by the coexistence of excess myoinositol. This fact tells us a possibility that at least in the liver, exogeneous myoinositol possesses some connections with phospholipid metabolism.

SUMMARY

Administration of a large excess myoinositol to young rats fed on Forker's basal diet provoked significant delay in growth and the rise of P/N ratio of hepatic lipid fraction. However, these effects were diminished by elevating the content of protein in the ration, or by increasing the amount of choline administration. The fact should be an additional evidence for the concept of antagonism between choline and myoinositol, and for that of the intervention of myoinositol into the phospholipid metabolism.

From the present result together with the previous one that proved a beneficial effect of myoinositol on the growth of rats, it was concluded that the exogenous myoinositol could be precursor of inositol phosphatide and promotes the cell growth so far as its amount is not increased enough to disturb the phospholipid metabolism e.g. choline phosphatide biosynthesis.

REFERENCES

1. Eagle, H., Oyama, V. I., and Freeman, A. E., *J. Biol. Chem.* **225,** 191 (1957).
2. Eagle, H., Agranoff, B. W., and Snell, E. E., *J. Biol. Chem.* **235,** 1891 (1960).
3. Lucas, G. H. W., *J. Physiol. Chem.* **28,** 1183 (1924).
4. Buston, H. W., and Pramanik, B. N., *Biochem. J.* **25,** 1656 (1931).
5. Beadle, G. W., and Tatum, E. L., *Amer. J. Botany* **23,** 678 (1945).
6. Daughaday, W. H., Lancer, J., and Haratnett, C., *J. Biol. Chem.* **212,** 869 (1955).
7. Imai, Y., *J. Biochem.* **53,** 50 (1963).
8. Eisenberg, F., Jr. and Bolden, A. H., *Biochem. Biophys. Res. Commun.* **12,** 72 (1963).
9. Freinkel, N., and Dawson, R. M. C., *Biochem. J.* **81,** 250 (1957).
10. Yagi, K., Kotaki, A., and Yamamoto, Y., *J. Vitaminol.* **11,** 14 (1965).
11. Agranoff, B. W., and Fox, M. R. S., *Nature* **183,** 1259 (1959).
12. Entenmann, C., Methods in Enzymol. Vol. III, p. 299 (1957).
13. Nakamura, M., *Nogei-Kagaku-Kaishi*, **24,** 1 (1950).
14. Allen, R. J. L., *Biochem. J.* **34,** 858 (1940).
15. Lubochinsky, B., and Zalta, J., *Bull. Soc. Chim. Biol.* **36,** 1363 (1954).
16. Zlatkis, A., Zak, B., and Boyle, A. J., *J. Lab. Clin. Med.* **41,** 486 (1953).
17. Agranoff, B. W., Bradley, R. M., and Brady, R. O., *J. Biol. Chem.* **233,** 1077 (1958)
18. Niwa, S., Kotaki, A., and Yagi K., *Vitamins* **30,** 23 (1964).
19. Forker, B. R., and Morgan, A. F., *J. Biol. Chem.* **209,** 303 (1954).
20. Nixon, D. A, *Nature* **187,** 77 (1960).
21. Hokin, L. E., and Hokin, M. R., *Nature* **184,** 1068 (1959).
22. Hayashi, K., Nakamura, I., and Yamazoe, S., *J. Biochem.* **56,** 381 (1964).
23. Hawthorne, J. N., and Kemp, P., *Adv. Lipid Res.* **2,** 127 (1964).
24. Vignais, P. M., Vignais, P. V., and Lehninger, A. L., *J. Biol. Chem.* **239,** 2011 (1964).
25. Hanahan, D. J., and Thompson, Jr., G. A., *Ann. Rev. Biochem.* **32,** 215 (1963).
26. Handler, P., *J. Nutrition* **31,** 621 (1946).
27. Jukes, T. M., *Poultry Sci.* **20,** 251 (1941).
28. Margolis, R. U., and Heller, A., *Biochim. Biophys. Acta* **98,** 438 (1965).
29. Petzold, G. L., and Agranoff, B. W., *Fed. Proc.* **24,** 476 (1965).

INOSITOL A TUMOR GROWTH INHIBITOR

THE importance of inositol for normal growth was established by the investigations of Eastcott,[1] Woolley[2] and others. There are no reports to date on the influence of inositol on malignant growth.

In this communication we describe the results of experiments dealing with the action of inositol on tumor growth. For these studies a rapid test for tumor growth inhibitors was employed.[3] In this test the inhibition of tumor growth is judged by comparing tumor sizes and tumor weights of treated groups of mice with untreated ones in an experimental period of 48 hours.

In Table 1 a series of experiments is presented, in which varying doses of inositol were studied. From this table it is evident that intravenous injections of inositol inhibit tumor growth, the degree of inhibition depending on the dose injected. Since September, 1942, inositol in varying doses was used in 16 experiments on 400 animals with the corresponding number of controls. The results of these experiments were similar to those presented in Table 1.

TABLE 1

EFFECT ON TUMOR GROWTH OF FOUR INTRAVENOUS INJECTIONS OF INOSITOL IN VARYING DOSES GIVEN OVER A PERIOD OF 48 HOURS*

Group No.	No. of animals in each group	Dose of Inositol γ	Mean terminal tumor weight mg	Standard error
453	11	0 (control: saline)	470	25.6
452	18	38	436	22.8
451	14	50	350	33.6
450	10	75	270	34.1
449	7	100	246	41.1
448	5	150	215	26.4
447	5	250	222	9.8
446	5	1000	142	12.8

*Female Rockland mice transplanted with Sarcoma 180; start of the experiment 8 days after transplantation; mice kept on polished rice diet for the experimental period of 48 hours.

Subcutaneous or oral administration of inositol was ineffective. Equally ineffective were intravenous injections of l-inositol,[4] inosose,[4] crystalline factors of the vitamin B-complex (thiamine, riboflavin, pyridoxine, nicotinamide, pantothenic acid, p-aminobenzoic acid, biotin and choline). Sodium phytate[4] and lipositol[4,5] showed an inhibition similar to that of inositol.

CONCLUSIONS

Inositol was found to inhibit tumor growth. The degree of inhibition depends on the dose injected. Inositol, a pure crystalline substance, can be used as a standard of reference for testing tumor growth inhibitory factors.

D. LASZLO
C. LEUCHTENBERGER

MOUNT SINAI HOSPITAL,
NEW YORK, N. Y.

[1] E. V. Eastcott, *Jour. Phys. Chem.*, 32: 1096, 1928.
[2] D. W. Woolley, SCIENCE, 92: 384, 1940; *Jour. Biol. Chem.*, 139: 29, 1941.
[3] D. Laszlo and C. Leuchtenberger, Cancer Research, 1943. To be published.
[4] We are indebted to Dr. D. W. Woolley, of the Rockefeller Institute, New York, for generously supplying us with these substances.
[5] D. W. Woolley, *Jour. Biol. Chem.*, 147: 581, 1943.

J. Biol. Chem. 159:605-609, 1945

THE DIETARY PRODUCTION OF FATTY LIVERS RESISTANT TO THE ACTION OF CHOLINE

By M. L. MacFARLAND and E. W. McHENRY

(From the School of Hygiene, University of Toronto, Toronto, Canada)

(Received for publication, May 14, 1945)

In 1940 it was reported from this laboratory (1) that the administration to rats of a beef liver fraction, in conjunction with several B vitamins, caused the production of fatty livers, high in cholesterol, resistant to the lipotropic action of choline, but preventable by lipocaic. Inositol was found to be lipotropic for the fatty liver thus produced (2). Subsequently it was stated (3) that the liver fraction could be replaced by biotin; at that time, however, practically all of the investigation was carried out with a relatively crude solution of biotin, pure biotin being available for one group of rats only. Pure biotin produced fatty livers containing large amounts of cholesterol and they appeared to resemble the ones caused by the beef liver fraction or by impure solutions of biotin. Consequently, this kind of fatty liver (resistant to choline) has been referred to as the "biotin" type. Recently we have had an opportunity to investigate more thoroughly the production of this kind of fatty liver. It would appear that pure biotin produces a fatty liver only partially resistant to choline and that the beef liver fraction contains a substance, other than biotin, which causes a more complete resistance to choline.

Methods

Rats were employed as test animals. The strain, age, and care were the same as previously described (4). To deplete the rats of their stores of B vitamins and of fat, they were maintained for 3 weeks on a fat-free, B vitamin-free basal diet (4). At the end of this period body weight and body fat had diminished markedly. During the following week various combinations of supplements were supplied to different groups. Thiamine, riboflavin, pyridoxine, calcium pantothenate, and biotin were given by subcutaneous injection. Supplements other than these were mixed with the food. The amounts of various supplements supplied per rat per day were as follows: thiamine hydrochloride (Merck) 25 γ, riboflavin (Merck) 25 γ, pyridoxine hydrochloride (Merck) 40 γ, calcium pantothenate (Merck) 100 γ, choline hydrochloride (Merck) 10 or 20 mg. as indicated, biotin (S. M. A.) 5 γ, inositol (S. M. A.) 10 or 20 mg. as indicated, and beef liver fraction (Connaught Laboratories) 2 cc. The liver fraction was similar to that used previously (5).

TABLE I

Effects of Liver Fraction and of Biotin upon Action of Lipotropic Agents

Rat group No.	Treatment during test wk.	Body weight change in test wk.	Liver weight	Crude fatty acids							Total cholesterol				
				Liver			Carcass			Total	Liver		Carcass		Total
						Iodine No.			Iodine No.						
		gm.	*gm.*	*mg. per liver*	*per cent*		*mg. per carcass*	*per cent*		*mg. per rat*	*mg. per liver*	*per cent*	*mg. per carcass*	*per cent*	*mg. per rat*
231	Depleted; no supplements		3.4	131	3.8	74	1200	1.9	77	1,331	8	0.23	190	0.30	198
226	B vitamins, liver fraction	+40	7.9	1585	20.1	63	8344	8.3	57	9,929	80	1.01	311	0.31	391
227	Same as Group 226 + 10 mg. choline	+42	8.0	1573	19.7	56	9344	9.0	64	10,917	92	1.15	280	0.27	372
229	" " " 226 + 20 " "	+40	8.3	1424	17.2	58	8352	8.4	65	9,776	79	0.95	259	0.26	338
228	" " " 226 + 10 " inositol	+42	5.9	268	4.5	69	9072	8.7	62	9,340	24	0.40	250	0.24	274
230	" " " 226 + 20 " "	+34	5.7	217	3.8	82	8054	8.2	67	8,271	19	0.33	246	0.25	265
232	" " " 226 + 10 " choline, 10 mg. inositol	+35	6.1	276	4.5	72	8309	8.6	64	8,585	21	0.31	249	0.26	270
252	B vitamins, biotin	+22	7.3	1827	25.0	59	5862	6.8	64	7,689	50	0.68	226	0.26	276
254	Same as Group 252 + 10 mg. choline	+23	5.4	537	10.0	66	7315	8.1	62	7,852	34	0.62	256	0.28	290
256	" " " 252 + 20 " "	+23	5.3	488	9.2	62	6718	7.5	65	7,206	33	0.62	251	0.28	284
253	" " " 252 + 10 " inositol	+21	5.7	875	15.5	62	5960	6.8	65	6,835	39	0.69	199	0.23	238
255	" " " 252 + 20 " "	+24	5.6	595	10.7	62	7084	7.8	63	7,679	33	0.60	221	0.24	254
257	" " " 252 + 10 " choline, 10 mg. inositol	+23	4.8	197	4.1	71	6912	7.5	64	7,109	14	0.30	227	0.25	241

The rats were anesthetized with nembutal, the livers removed, and the total crude fatty acids in the livers and bodies were determined by methods previously published (4, 6). The total cholesterol content of the livers and bodies was measured by means of the procedure of Schoenheimer and Sperry (7) and the iodine numbers of the crude fatty acids were determined by the method of Yasuda (8). All reported results are averages for groups of ten rats.

Results

The effects of a beef liver fraction in causing fatty livers in rats have been repeatedly confirmed in this laboratory. For the present purpose it was advisable to demonstrate the effect again under conditions comparable to those in which biotin was used. The experiment with biotin was repeated twice and entirely similar results were secured on each occasion; for brevity only one set of results is given. Table I shows the data secured by the use of beef liver fraction and with biotin.

DISCUSSION

As in a number of similar experiments, the administration of the beef liver fraction, in conjunction with several B vitamins, caused the production of acutely fatty livers in rats maintained on a high carbohydrate, fat-free ration. There was evidence of synthesis of large amounts of fat and of considerable cholesterol. Choline, at the dosage levels used, caused negligible changes in the concentration and in the absolute amounts of fat and of cholesterol in the liver. Given in the same amounts as used for choline, inositol produced marked reductions in liver fat and cholesterol. The effect of a combination of choline and inositol is similar to that produced by the same amount of inositol alone. It is obvious that inositol is effective in preventing the fatty liver caused by the beef liver fraction, while choline is not.

In the case of three similar experiments, one of which has been reported above, biotin has produced acutely fatty livers, but the cholesterol is less than when liver fraction was supplied. Both choline and inositol are partially effective as lipotropic agents for the fatty livers produced with biotin and a combination of the two is much more effective than like amounts of the two substances given separately. As judged by the action of these lipotropic agents, the fatty liver caused by biotin is definitely different from that obtained with the beef liver fraction. The term "biotin fatty liver" should be restricted and is not applicable, as we had assumed previously (9), to fatty livers caused by the beef liver fraction.

In an earlier paper (2) attention was drawn to the action of inositol causing a marked decrease in liver cholesterol. Such is the case when li-

fraction is supplied but in the biotin experiments both choline and inositol exerted an effect upon the cholesterol content of the liver. Reference has been made (1) to the synthesis of cholesterol which occurs in experiments such as the present one. It is not clear whether a particular constituent of the diet promotes the synthesis of cholesterol or whether cholesterol is formed as an accompaniment to fat synthesis. It is also doubtful whether a particular lipotropic agent causes the removal of cholesterol from the liver or whether the amount of cholesterol is reduced because it is removed with fatty acids. In the biotin experiment the second alternative seems plausible. Beveridge and Lucas (10) reported that, under the conditions used by them, choline produced greater decreases in liver cholesterol than did inositol. It would appear that liver cholesterol is reduced when the total amount of fat is diminished and that a lipotropic agent is effective for cholesterol when it definitely reduces the amount of fatty acids in the liver.

An explanation for the difference in the actions of the two lipotropic factors cannot be supplied at present. Some of the conditions which alter the response were indicated in a report from this laboratory in 1943 (11). Choline was found to be effective for "thiamine fatty livers," while inositol was not; inositol was active for "biotin fatty livers" (a term then incorrectly used), while choline was inactive. It would appear that inositol becomes effective when several B vitamins are supplied. Recently Beveridge and Lucas have reported (10) that the inclusion of corn oil in the diet prevents inositol from exerting a lipotropic effect. It was suggested in a review from this laboratory (12) that choline will combine with certain fatty acids, while inositol selects quite different ones. It should be noted that the iodine number of the crude fatty acids in the liver is somewhat greater when liver fraction is given than when biotin is supplied. When inositol is fully active (as in Rat Group 230), the iodine number of the liver fatty acids is definitely increased. This can hardly be taken to mean that inositol has combined with saturated fatty acids and caused their removal from the liver, since there appears to be an increase in the iodine number of liver fatty acids whenever a lipotropic agent has been active and has produced a turnover of fatty acids. A comparison of the fatty acids present in the livers of rats with and without a supply of beef liver fraction (13) showed that the liver fraction causes a decrease in the concentration of saturated fatty acids and an increase in unsaturated ones, particularly of oleic acid. A similar study has not yet been made on the livers of rats given biotin.

The constituent of the liver fraction which causes the alteration in the response to the lipotropic factors is unknown at present. The liver fraction is known to contain most of the B vitamins as well as folic acid and other

substances. On the basis of previous work (11) many of these can be eliminated as causative agents (thiamine, riboflavin, pyridoxine, pantothenic acid). It should be pointed out that the liver fraction contains no cholesterol. Attempts to identify the active constituent are in progress.

SUMMARY

The administration of a beef liver fraction to rats maintained on a high carbohydrate, fat-free diet causes the production of fatty livers resistant to choline, but the liver fat can be reduced to low levels by supplying inositol. The use of biotin in place of the liver fraction causes fatty livers which are partially responsive to either lipotropic agent and completely prevented by a conjoint supply of choline and inositol.

This investigation was made possible by grants from the Division of Natural Sciences of the Rockefeller Foundation and from the Connaught Laboratories, University of Toronto.

BIBLIOGRAPHY

1. McHenry, E. W., and Gavin, G., *Science*, **91**, 171 (1940).
2. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 485 (1941).
3. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **141**, 619 (1941).
4. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **132**, 41 (1940).
5. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **134**, 683 (1940).
6. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **125**, 653 (1938).
7. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
8. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).
9. McHenry, E. W., Biological symposia, Lancaster, **5**, 177 (1941).
10. Beveridge, J. M. R., and Lucas, C. C., *J. Biol. Chem.*, **157**, 311 (1945).
11. Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, **148**, 275 (1943).
12. McHenry, E. W., and Patterson, J. M., *Physiol. Rev.*, **24**, 128 (1944).
13. Longenecker, H. E., Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **139**, 611 (1941).

Am. J. Phy. 136:124-127, 1942

THE INTERRELATIONSHIP OF p-AMINOBENZOIC ACID AND INOSITOL

GUSTAV J. MARTIN

From the Warner Institute for Therapeutic Research, 113 West Eighteenth Street, New York City

Accepted for publication January 6, 1942

The discovery of the chromotrichial action of p-aminobenzoic acid by Ansbacher (1) precipitated a controversy as to the true nature of the grey hair factor or factors. Unna et al. (2) and Emerson (3) failed to find any chromotrichial action attributable to p-aminobenzoic acid and believe that pantothenic acid is the only grey hair factor. Martin and Ansbacher (4) noted a chromotrichial action of p-aminobenzoic acid in mice. It seemed obvious that some factor must be at play not recognized by either group of investigators. To bring these various observations into harmony has been the objective of this work.

PROCEDURE AND RESULTS. The basic diet used throughout these experiments consisted of:

	per cent
Vitamin free casein	18.0
Sucrose	67.0
Salts	4.0
Butter fat	9.0
Cod liver oil	2.0

The diet was made up each week, and the vitamin supplements were added as follows:

	mgm. per kilo of diet	*daily dose per rat*
Thiamine hydrochloride	5.0	50 μg.
Riboflavin	10.0	100 μg.
Pyridoxine	5.0	50 μg.
Nicotinic acid	100.0	1 mgm.
Calcium pantothenate	100.0	1 mgm.
Choline chloride	200.0	2 mgm.
Inositol	200.0	2 mgm.
p-Aminobenzoic acid	100.0	1 mgm.

Each experimental group consisted of 30 Rockland strain black rats.

Diet 1. Inositol and pantothenic acid deficiency: This diet contained six factors but was deficient in pantothenic acid and in inositol. On this combined deficiency, the Rockland strain black rat attains a weight of approximately 55 to 65 grams and death occurs within five or six weeks. Spectacled eyes are seen within two weeks. The involvement of the eyes develops to a pan-ophthalmitis. This is bilateral and severe. Purulent exudate, bloody exudate, rupture of

eyeballs and complete blindness characterize the syndrome. It is far more marked than we have noted in a straight pantothenic acid deficiency or in a straight inositol deficiency. There is some alopecia of a very mild variety. Greying or achromotrichia is not a pronounced manifestation. The animals are a brownish grey. The fur has a wet, oily appearance. These animals before death invariably show a peculiar gait; the hind legs are handled awkwardly and do not seem to respond in coördination with the forelegs. Complete paralysis was not seen in any of the thirty animals in this group but fully 90 per cent showed this spasticity.

Diet 2. Inositol deficiency: This diet contained seven of the factors listed and was deficient in inositol. The growth rate of this set is such that after four or five weeks the average weight is 70 to 90 grams. At five weeks the animals show a pattern greying which is markedly similar to that of a pantothenic acid deficiency. Spectacled eyes are common, with pan-ophthalmitis occurring infrequently and not to the marked degree seen in a combined inositol and pantothenic acid deficiency. Again, in this group the locomotor incoördination occurs, but to a lesser degree; and the mild alopecia observed might be called rather a thinning of the hair than real alopecia. The syndrome associated with inositol deficiency is difficult to distinguish from that of a pantothenic acid deficiency.

Diet 3. p-Aminobenzoic acid deficiency: Eighty per cent of the animals were dead within six weeks. The weights of this set reached 50 to 60 grams. The appearance is most unusual: the hair over the body is sleek and black and appears darker than its original color; the hair over the head is sparse, with a mild greying. Crusted, bloody, scaly feet were frequently seen. These animals seem to have no energy; they move sluggishly with hunched backs.

Diet 4. Pantothenic acid deficiency: No deaths occurred in this group during the first eight weeks. The weights attained averaged 70 to 100 grams. A typical pattern greying was seen, accompanied by the usual spectacled eyes. A mild form of alopecia was noted. Pan-ophthalmitis was seen in but two of the thirty rats on this pantothenic acid deficient diet.

Diet 5. This diet contains the eight members of the B complex listed. At six weeks, the weight attained averaged 110 to 180 grams. The animals seemed normal in every respect except that the coat was rather brownish. This diet was supplemented with wheat germ oil, ethyl linoleate and biotin, and found to be complete as evidenced by a failure of biotin or the essential unsaturated fatty acids to increase the growth rate.

Diet 6. This diet contained no inositol and no p-aminobenzoic acid, but did contain the other six factors as listed. These rats do not differ in any way from those on diet 5. The weight, the appearance, in every respect, they are the same. Thus, in the absence of both inositol and p-aminobenzoic acid, the six factors, namely, thiamine, riboflavin, pyridoxine, choline, nicotinic acid and calcium pantothenate are adequate.

Discussion. The results of Unna et al. (2) and Emerson (3) are confirmed in that the six basic factors (thiamine, riboflavin, nicotinic acid, pyridoxine, choline and calcium pantothenate) are adequate for seemingly normal nutrition.

They are not adequate if either p-aminobenzoic acid or inositol is added to the diet. Ansbacher (1) and Martin and Ansbacher (4) had inositol in their basic supplements and thus p-aminobenzoic acid deficiency is noted. Neither Unna et al. (2) nor Emerson (3) had inositol in the basic diet used. It is possible that the explanation lies in a stimulation and/or inhibition of bacterial growth in the intestinal tract and hence the bacterial synthesis of vitamin factors, known or unknown in nature. Both factors, namely, inositol (5) and p-aminobenzoic acid (6, 7), have been demonstrated as growth factors for yeast or bacteria, and p-aminobenzoic acid has been demonstrated to inhibit bacterial growth (8) at certain concentrations. The utilization and consequent destruction of various vitamins by microörganisms has been demonstrated (9, 10). Further, the synthesis of certain factors, particularly biotin (11) and inositol (12) by organisms present in the gastro-intestinal tract has been reported. The stimulation of the growth of microörganisms by one member of the B complex causing an increased synthesis of another member of the B complex by that same organism is not only probable but certain. The analysis we place on the above reported results is that inositol stimulates the growth of organisms which utilize and destroy some member of the B complex, known or unknown, thus precipitating a deficiency of that factor. p-Aminobenzoic acid either through stimulation or inhibition of bacterial growth precipitates an inositol deficiency (4). This seems the logical explanation, in view of the fact that the elimination of both inositol and p-aminobenzoic acid from the diet, feeding only six of the B complex factors results in a state of nutrition which is the equal of that noted if the eight factors are included.

The addition of inositol to a diet may stimulate the growth of organisms which destroy pantothenic acid, whereas the addition of p-aminobenzoic acid may inhibit the growth of these organisms. This explanation can be advanced even if inhibition is not considered, as specific stimulation may cause an overgrowth of one type of organism and the consequent elimination from the gastro-intestinal tract of another. In the preliminary experiments, it is our opinion that proteus vulgaris is absent from the gastro-intestinal tracts of rats fed excessive amounts of p-aminobenzoic acid (5 mgm. daily). Further, we have noted a tendency on the part of the acid forming organisms to overgrow. Thus, we see both an apparent inhibition of proteus and a stimulation of the lactic acid forming organisms. The seeming inhibition of proteus may be actually due to overgrowth on the part of the acid forming organisms.

It is not desired to suggest an alteration of the status of either inositol or p-aminobenzoic acid as a member of the B complex, but to point out that one precipitates a deficiency of the other under our experimental conditions.

The addition of p-aminobenzoic acid to the basic diet made it possible for Martin and Ansbacher (4) to produce an inositol deficiency, where in the absence of p-aminobenzoic acid no inositol deficiency developed (13). Woolley, who originally described this syndrome (14), stated subsequently that in many instances spontaneous cures occurred (15). It is these spontaneous cures which are prevented by the p-aminobenzoic acid. The cures were due to synthesis of

inositol by organisms present in the gastro-intestinal tract (12), and it seems probable that it is this synthesis which is prevented by p-aminobenzoic acid.

CONCLUSIONS

Six B complex factors (thiamine, riboflavin, pyridoxine, choline, nicotinic acid and calcium pantothenate) afford seemingly normal nutrition to the Rockland strain black rat. Addition of inositol precipitates a syndrome prevented by p-aminobenzoic acid. Addition of p-aminobenzoic acid precipitates a syndrome prevented by inositol. Eight B complex factors (the six listed above plus inositol and p-aminobenzoic acid) afford seemingly normal nutrition to these rats.

REFERENCES

(1) ANSBACHER, S. Science **93**: 164, 1941.
(2) UNNA, K., G. V. RICHARDS AND W. L. SAMPSON. J. Nutrition **22**: Dec. 10, 1941.
(3) EMERSON, G. A. Proc. Soc. Exper. Biol. and Med. **47**: 448, 1941.
(4) MARTIN, G. J. AND S. ANSBACHER. Proc. Soc. Exper. Biol. and Med. **48**: 118, 1941.
(5) EASTCOTT, E. V. J. Physiol. Chem. **32**: 1094, 1928.
(6) LAMPEN, J. O. AND W. H. PETERSON. J. Am. Chem. Soc. **63**: 2283, 1941.
(7) RUBBO, S. P. AND J. M. GILLESPIE. Nature **146**: 838, 1940.
(8) MARTIN, G. J. AND C. V. FISHER. Unpublished data.
(9) PELCZAR, M. J. AND J. R. PORTER. J. Bact. **39**: 429, 1940.
(10) SNELL, E. E. AND W. H. PETERSON. J. Bact. **39**: 273, 1940.
(11) LANDY, M. AND D. DICKEN. J. Bact. **41**: 40, 1941.
(12) WOOLLEY, D. W. Proc. Am. Soc. Bact., Baltimore.
(13) MARTIN, G. J. Science **93**: 422, 1941.
(14) WOOLLEY, D. W. J. Biol. Chem. **136**: 113, 1941.
(15) WOOLLEY, D. W. Proc. Soc. Exper. Biol. and Med. **46**: 565, 1941.

Am. J. Digestive Diseases 8:290-295, 1941

The Influence of Inositol and Other B Complex Factors Upon the Motility of the Gastro-Intestinal Tract

By

GUSTAV J. MARTIN, Sc.D., MARVIN R. THOMPSON, Ph.D.

and

JOSE DE CARVAJAL-FORERO, B.S.S., Ph.B.*

NEW YORK, NEW YORK

THE most recently identified member of the B complex is inositol. The symptomatology of a deficiency of this substance has been described by two investigators (1, 2) and has been observed in these laboratories (3). Norris and Hauschildt (1) supplemented a purified diet with thiamine, pyridoxine, nicotinic acid and riboflavin and observed in mice on this diet failure of growth and the development of a deficiency dermatitis. Simultaneously, Woolley (2) described this syndrome using as basic supplements thiamine, riboflavin, nicotinic acid, pyridoxine, β-alanine, pantothenic acid and choline. All investigators (1, 2, 3) have noted essentially the same

*Warner Institute for Therapeutic Research, 113 West 18th Street, New York City, N. Y.

Submitted February 15, 1941.

Fig. 1. Two hours after ingestion of plain Barium Sulfate. Note small gastric residue, irregular distribution and head of barium column in splenic flexure.

symptoms. The hair on the entire body excepting the head and tail falls out, leaving the trunk naked. The denuded areas show a reddening, and some sores develop. Obvious manifestations of nervous involvement are seen. Woolley (4) isolated the factor and identified it as phytin or inositol, phytin being the calcium magnesium salt of inositol phosphoric acid. Inositol itself has been of interest since the report of Eastcott (5) showing that inositol was bios 1, a substance necessary for the normal reproduction of the yeast cell. It is to be noted that it is the meso form of inositol which functions as bios.

Our observations on inositol deficient mice led us to believe that constipation was a characteristic finding. This combined with statements found in the literature (6, 7) to the effect that phytin and inositol are not biologically inert substances, led us to investigate the effect of inositol on the gastro-intestinal tract.

EXPERIMENTAL

Dogs maintained on a mildly constipating diet were used throughout this work. The animals were kept for two days before use on a diet containing 30% albumin and 0.1% of agar. This balance between albumin and agar produces a constipation of a very mild degree. The diet contained 10% yeast, and it is assumed that no deficiencies of B complex members existed. From a nutritional standpoint, the animals were normal.

A roentgenological study of the physiological effect upon the gastro-intestinal tract of dogs, which had been carefully conditioned on the constipating diet described above, was carried out using for each series of four dogs one of the following mixtures:

Series A: Control Series; Plain Barium Sulfate.

Series B: Barium Sulfate with Inositol.

Series C: Barium Sulfate with thiamin hydrochloride, riboflavin, pyridoxine, calcium pantothenate and choline.

Series D: Barium Sulfate with nicotinic acid.

Series F: Barium Sulfate with thiamin hydrochloride, riboflavin, pyridoxine, calcium pantothenate, choline and nicotinic acid.

Series G: Barium Sulfate with Inositol and nicotinic acid.

The results observed for each series may be summarized as follows:

Series A: Control Series; Plain Barium Sulfate. There was a rather slow peristalsis in the stomach; barium started to pass into the duodenum in from 7 to 10 minutes with an average of 9 minutes for the four dogs. At the end of the first hour the stomach was about half empty with the head of the barium column in the ileum. Under the fluoroscope several loops of the jejunum were seen rather dilated with corresponding constrictions. A spastic type of peristalsis of the small intestine was observed. At the end of the second hour the stomach was almost empty, the head of the barium column having progressed to the large intestine, a small amount as far as the splenic flexure (see Fig. 1). Peristalsis was observed as before. At the end of the third hour there was still a very small gastric residue; the head of the barium was in the splenic flexure, although in one dog it had reached the sigmoid, and a large amount was present in the ileum. At the end of the fourth hour the small gastric residue was still present. The head of the barium column filled the large intestine up to the splenic flexure more uniformly with the exception of the dog mentioned above. Some barium was still present in the terminal loops of the ileum. At the end of the fifth hour improved filling of the large intestine

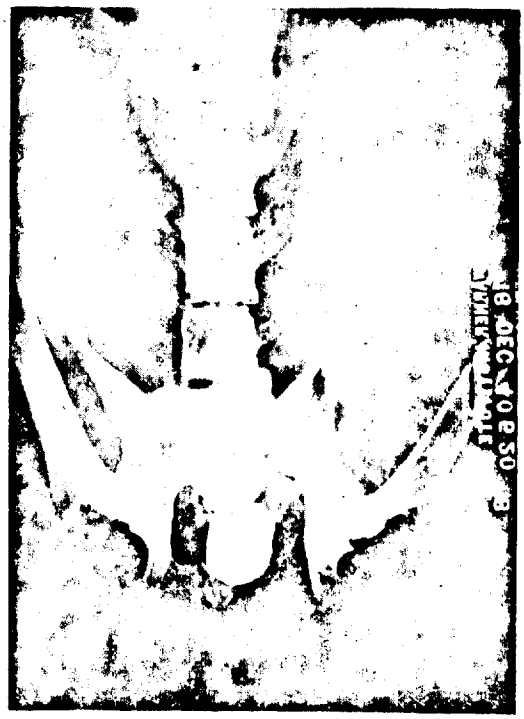


Fig. 2. Same subject as in Fig. 1: forty-eight hours after ingestion of plain Barium Sulfate. Note small residue in sigmoid and rectum.

Fig. 3. Two hours after ingestion of Barium Sulfate mixed with Inositol. Note almost complete emptying of the small intestine and thorough filling of the colon.

to the rectum was observed; there was still retention in several loops of the terminal ileum. At the end of the sixth hour there was practically no change from the preceding radiogram. At the end of twenty-four hours there was observed a partial evacuation of the colon with some barium retained in the sigmoid and rectum. In two dogs, the retention extended to the distal transverse colon. At the end of forty-eight hours, there was a very small residue in the rectum (see Fig. 2).

Series B: Barium Sulfate with Inositol (20 mg./kilo.). This treatment produced a very active peristalsis of the stomach with deep peristaltic waves coming rapid. There was a definite pyloro-spasm; barium began to pass into the duodenum in from 10 to 15 minutes with an average of 13 minutes. At the end of the first hour the stomach was two-thirds empty, with the barium regularly distributed throughout the small intestine. Peristalsis of the small intestine was rhythmical with no constrictions nor dilatations observed. The barium meal progressed rather rapidly. At the end of the second hour the stomach was almost completely empty, the head of the barium column in one dog was in the rectum, the entire colon being completely filled (see Fig. 3). In the other three dogs there was a very regular distribution of the material throughout the small intestine, the head of the barium meal having reached the caecum. At the end of the third hour there was no change in the dog whose colon was entirely filled. In two of the others the head of the barium had reached the sigmoid; while in the fourth there was a massing of the meal in the terminal loops of the ileum. At the end of the fourth hour there was no difference from the previous radiogram with the exception of the fourth dog in which the head of the column was in the descending colon. At the end of the fifth hour there was practically no change from the preceding radiogram. At the end of the sixth hour there was no marked change. At the end of the twenty-fourth hour there was a complete evacuation in one of the subjects (see Fig. 4) and almost complete in two others. The fourth still showed some retention in the descending colon, sigmoid and rectum. At the end of the forty-eighth hour the colon was entirely empty in all subjects.

Series C: Barium Sulfate with thiamine hydrochloride, riboflavin, pyridoxine, calcium pantothenate and choline (20 mg./kilo. of each). There was an active peristalsis of the stomach, barium starting to pass into the duodenum in from 6 to 7 minutes with an average of six and a half minutes. At the end of the first hour the stomach was three-quarters empty. The barium meal was distributed very regularly throughout the small intestine; the peristalsis of this intestine was slightly spastic although there were not as marked constrictions and dilatations as when barium alone was administered. At the end of the second hour there was a small gastric residue, the head of the barium column having reached the rectum, although there was quite a large amount in the terminal ileum (see Fig. 5). At the end of the third hour a slight advance in the large intestine was present; some barium still being retained in the terminal loops of the ileum. At the end of the fourth and fifth hours there was very little change. At the end of the sixth hour the entire large intestine was completely filled. At the end of the twenty-fourth hour the intestine was completely empty.

Series D: Barium Sulfate with nicotinic acid (20 mg./kilo.). Two series were made with the above combination. In the first a large amount of nicotinic acid was added to the barium; in the second, half of

Fig. 4. Same subject as in Fig. 1, 2, 3. Twenty-four hours after ingestion of Barium Sulfate mixed with Inositol. Note entire emptying of colon.

the original amount (10 mg./kilo) was added. In the first series the stomach showed a very flat shallow peristalsis, there being practically no motion of the stomach. At the end of 12 to 15 minutes, peristalsis became apparent and barium began to pass into the duodenum within three minutes. In the second series peristalsis was active; there was a complete absence of pyloro-spasm with the barium passing into the duodenum in an average of three minutes. This passage, however, was rather slow as the peristaltic wave was not very deep. At the end of the first hour, the stomach was about one-half empty. The head of the barium in the jejunum and the peristalsis of the latter was rather sluggish. At the end of the second

Fig. 5. Same subject as used in Fig. 1, 2, 3, 4. Two hours after ingestion of Barium Sulfate mixed with components of series C. Note small gastric residue, head of barium column in rectum, retention in terminal ileum and slight spasticity of ileum.

hour the stomach was empty in the second series; half full in the first series. The entire small intestine was completely filled. Peristalsis of the intestine was still sluggish. At the end of the third hour, the stomach was one-third full in the first series and empty in the second series. The distribution was quite irregular throughout the small intestine with small amounts in the large intestine. At the end of the fourth hour there was an improved filling of the large intestine in the first series, with a small residue in the terminal loops of the ileum; while in the second series the distribution was irregular with the head of the barium column in the proximal descending colon and a large amount retained in the terminal loops of the ileum. At the end of the fifth hour there was no very marked change from the preceding radiogram. At the end of the sixth hour there was a partial evacuation in one of the dogs of the first series. In the other dog of this same series the colon was well filled reaching the sigmoid. In the second series the head of the colon was at the middle of the descending colon. At the end of the twenty-fourth hour one dog showed a complete evacuation while the others showed a considerable retention in the descending sigmoid and rectum.

Series F: Barium Sulfate, thiamine hydrochloride, riboflavin, pyridoxine, calcium pantothenate, choline and nicotinic acid (20 mg. kilo. of each). There was an excellent peristalsis of the stomach. Barium passed into the duodenum in three minutes although the passage was not very copious. At the end of the first hour the stomach was half empty, barium was distributed throughout the jejunum, and the peristalsis of this part of the small intestine was rather sluggish. At the end of the second hour the stomach was almost completely empty. The head of the barium column had reached the large intestine in one case as far as the sigmoid although the distribution was generalized with a large amount still present in the ileum. Peristalsis of the small intestine was somewhat more active than previously. At the end of the third hour the head of the column had reached the rectum. The large intestine was fairly well filled although there was a small amount still retained in the terminal loops of the ileum. At the end of the fourth hour there was practically no change from the preceding radiogram. At the end of the fifth hour there was a better filling of the large intestine with traces in the terminal loops of the ileum. At the end of the sixth hour a small partial evacuation had taken place although barium was still present throughout the entire large intestine. At the end of the twenty-fourth hour some more barium had been evacuated, but the residue left in the large intestine exceeded half. At the end of the forty-eighth hour there was still a small amount of barium present in the sigmoid and colon.

Series G: Barium Sulfate with inositol and nicotinic acid. This series was further subdivided: in one, equal amounts of inositol (20 mg./kilo.) and nicotinic acid (20 mg./kilo.) were mixed with the barium, in the other one-half of this amount (10 mg./kilo.) of nicotinic acid was administered with the inositol at the same level. In series G-1 peristalsis of the stomach was very active. Deep peristaltic waves were observed, but there was a definite pyloro-spasm. Barium began to pass into the duodenum at the end of from 12 to 15 minutes for an average of 13 minutes. In Series G-2 peristalsis of the stomach was very active, and pyloro-spasm completely absent. Barium began to pass into the duodenum at the end of from 2 to 4 minutes with an average of three minutes. At the end of the first hour the stomach was three-quarters empty in G-1 while it was almost completely empty in G-2. There was a better peristalsis of the small intestine in the G-1 series than there was in the G-2 series. In the latter the intestine seemed rather sluggish. At the end of the second hour there was a continued active peristalsis in the small intestine in series G-1. The head of the barium column had reached the sigmoid in one subject and the ascending colon in the others. In series G-2 the sluggishness persisted, and the barium was not as far advanced. At the end of the third hour there was a better filling of the large in-

testine in series G-1 although there was some scattered in the terminal ileum. In series G-2 there was an improvement in the peristalsis of the intestine, and the head of the barium column was seen in the descending colon. At the end of the fourth hour there was some advance in the progress of the barium, in both series the colon was better filled, and there was a slight segmentation observed. At the end of the fifth hour there was no appreciable change. At the end of the sixth hour there was a partial evacuation in series G-2, segmentation still persisting in this series. In series G-1 the entire colon was filled, with slight segmentation observed. At the end of the twenty-fourth hour the intestine was empty in series G-2 while there was a small residue in series G-1.

SUMMARY

From the above we feel justified in concluding that inositol has a definite action upon the stomach and small intestine. It markedly increases the peristaltic action of these organs without creating a spastic condition. The pylorospasm observed when inositol was administered may have been due in part to the constipating diet and in part to the fact that inositol may have a contracting effect upon the pyloric sphincter.

Nicotinic acid on the other hand decreases the peristaltic action of the stomach and small intestine. It produces a marked state of repose of these organs. Thiamine hydrochloride, riboflavin, pyridoxine, calcium pantothenate and choline by themselves and in combination with nicotinic acid did not materially affect the motility of the intestinal tract.

The action of the above upon the large intestine is not very marked although by the use of inositol the colon fills rather rapidly, and evacuation takes place quite completely when it occurs. There was no segmentation nor retention of material. The constipating diet alluded to may have obscured the physiological effects of this preparation upon the colon.

In order to obtain information as to the mode of action of inositol in the production of hypermotility of the stomach and small intestines, three dogs were given 100 mg. per kilo. doses while blood pressure determinations were being made. One of these animals received a total of 1.1 grams of inositol intravenously in a period of less than five minutes. This dog showed no variations in blood pressure, respiration or heart action. By these observations sympathetic and parasympathetic action has been excluded as the mode of action.

DISCUSSION

Comprehensive investigations have been made on the effect of B complex deficiency on the gastro-intestinal tract; and many of the individual members of the complex have been implicated in disturbed gastro-intestinal physiology.

The literature on thiamine indicates that the gastro-intestinal changes associated with thiamine deficiency have not been completely demonstrated as having direct origin in the deficiency of this vitamin. Most of the studies so far reported have been complicated by inadequacies of other factors of the B complex. Cowgill (8) has discussed this subject, and he points out that conditions such as amebic dysentery, commonly found in beriberi localities, may constitute the precipitating cause of the intestinal manifestations. This investigator (9) first noted anorexia in Vitamin B_1 deficiency and demonstrated hypochlorhydria and achlorhydria in dogs on a deficient diet (10). It is to be emphasized that the functional disturbances of the gastro-intestinal tract are much more severe than can be accounted for on the basis of demonstrable anatomical lesions. Alvarez, et al (11) and Sure, et al (12) have been unable to demonstrate any change in gastric secretion due to a lack of Vitamin B_1. Williams and Spies (13) state that thiamine is essential for the normal functioning of the gastro-intestinal tract. In some cases the motility is abnormal. There are no specific lesions demonstrable.

Sparks and Collins (14) have shown that thiamine deficiency in rats causes a marked increase in the volume of the colon. This demonstrates that thiamine has some direct action in maintaining intestinal tone. Babkin (15) has reported some interesting observations suggesting a relation of some member of the B complex to the nervous mechanism controlling gastric secretion. Dogs and cats suitably operated so as to permit experiments of the sham feeding type were allowed to subsist on a diet deficient in B complex and then were tested for their gastric responses to sham feeding, subcutaneous injections of histamine and the presence of food and 5% alcohol solution in the intestine. During the state of vitamin deficiency there was a marked diminution in the response of the gastric glands to these stimuli. When yeast was administered, the responses became normal within a few hours.

Chatterjee (16) studied the motor functions of the intestine in the presence of a B_1 deficiency. In the vitamin-deprived animals there was a definite decrease in the amplitude, the number and the intensity of intestinal contractions and responses to pilocarpine, atropine, nicotine and barium chloride.

Molitor and Sampson (17) have tested the influence of pure thiamine on intestinal motility, using isolated rabbit's intestine suspended in Ringer's solution as well as taking observation on the intestine in situ. The addition of pure vitamin in the in situ experiments was without any effect on the movements. Evidently, this substance has no demonstrable effect on the intestine of the normal organism.

Recently, Elsom, et al (18) maintained patients on a diet deficient in the B complex and studied among other things the gastro-intestinal tract. They noted mild anorexia after one week on the diet; this ultimately became extreme. Abdominal distension and constipation were marked. Mild soreness of tongue was an occasional complaint. As the deficiency became more pronounced nausea and vomiting were frequent, and during the last few days abdominal pain was noted. Appetite returned promptly following the administration of thiamine. This return of appetite was not associated with any change in the other gastro-intestinal symptoms except that abdominal pain disappeared. Toward the end of the second week of thiamine therapy appetite again decreased and, in spite of continued administration of thiamine and the addition of riboflavin, did not again return to normal until yeast was given. Roentgen examination of the gastro-intestinal tract at the end of the deficiency showed no abnormality except some increased caliber of jejunal loops. After the subject had received thiamine for 18 days there was delayed gastric

emptying and slight delay in small bowel motility. Increased caliber of the jejunal loops was still evident. Riboflavin did not significantly alter these findings. Following the administration of yeast, however, there was marked improvement in the small intestinal motility, the head of the barium column having reached the hepatic flexure four hours and forty minutes after the ingestion of the barium, while under thiamine and riboflavine therapy a comparable film was not observed until six hours after the start of the examination. A slight delay in gastric emptying time persisted.

He observed no segmented and dilated loops of small bowel which had been reported by Pendergrass and Comroe (19), Mackie and Pound (20) and Snell and Camp (21).

Indigestion and diarrhea have long been known as characterizing pellagra. Crandall, et al (22) state that a P-P factor (nicotinic acid, nicotinamide or a substance capable of replacing it) is essential for the maintenance of normal gastro-intestinal motility. Animals on a nicotinic acid deficient, black tongue diet, showed hypermotility of the gastro-intestinal tract. In 3 dogs barium reached the rectum in 2 hours. Thiamine and riboflavin were reported ineffective in functional digestive disturbances. The effect of nicotinic acid in correcting hypermotility is in complete harmony with our findings which clearly show that this acid decreases peristalsis in both the stomach and in the small intestine.

The results of Elsom, et al (18) are in complete harmony with the results reported in this paper. They noted that thiamin and riboflavin did not alter the delayed small bowel motility nor the delayed gastric emptying time seen in a B complex deficiency. Both conditions were corrected by the administration of yeast. Our results show that inositol is responsible for small bowel motility, and nicotinic acid and inositol function in gastric emptying time. Both were present in the yeast used by Elsom and his co-workers (18) and account for the results they record following the administration of yeast.

The effects observed in this study show clearly that the administration by oral routes of at least two of the known members of the B complex affects the gastro-intestinal tract of the nutritionally normal animal. As to the mode of action, it is not via the sympathetic or parasympathetic nervous systems and, therefore, probably depends upon direct stimulation of the musculature of the tract. Intravenous administration of inositol to dogs produces the same action as does oral administration, namely, a tendency to pylorospasm and increased motility of both the stomach and the small intestine. It is suggested that following intravenous dosage the compound is excreted into the alimentary canal and there brings about its characteristic action. In our earlier studies no inositol action on the large intestine was noted, but following larger doses a slight action is seen. Smaller doses are absorbed or destroyed by bacterial action before reaching the large gut.

Inositol is probably not a dietary essential for man. However, it is essential physiologically whether synthesized or of dietary origin, and one of its functions is control of the motility of the gastro-intestinal tract.

CONCLUSIONS

Inositol markedly increases the peristalsis of the stomach and the small intestine. Nicotinic acid decreases the peristaltic action of the stomach and small intestine. The other members of the B complex available in pure form, thiamine, riboflavin, choline, pyridoxine and pantothenic acid, have no apparent action of this type.

Inositol and nicotinic acid are the members of the Vitamin B complex directly concerned with gastro-intestinal motility, and it is suggested that the balance or ratio of nicotinic acid or similar compounds to inositol is the nutritional factor which determines hypo- or hypermotility.

REFERENCES

1. Norris, Earl R. and Hauschildt, J.: A New Factor of the Vitamin B Complex Required by Albino Mouse. *Science*, 92:316, 1940.
2. Woolley, D. W.: A New Dietary Essential for the Mouse. *J. Biol. Chem.*, 136:113, 1940.
3. Martin, Gustav J. and Hueper, W. C.: The Mouse Anti-alopecia Factor. (In press).
4. Woolley, D. W.: The Nature of the Anti-alopecia Factor. *Science*, 92:394, 1940.
5. Eastcott, Edna V.: Wilder's Bios. Isolation and Identification of Bios I. *J. Phys. Chem.*, 32:1094, 1928.
6. Anderson, R. J. and Bosworth, A. W.: Utilization of Inositol in Animal Organism. Effect of Inositol on Metabolism of Man. *J. Bio. Chem.*, 25:399, 1916.
7. Burns, David: Note on Effect of Purgation on the Creatinine Content of Urine. *Biochem. J.*, 14:94, 1920.
8. Cowgill, G. R.: The Vitamin B Requirement of Man. Yale U. Press, New Haven, 1934.
9. Cowgill, G. R.: Studies in Physiology of Vitamins. *Proc. Soc. Exp. Bio. and Med.*, 18:148, 1921.
10. Cowgill, G. R. and Gilman, A. Z.: Vitamins—Effect of Lack of Vitamin B Complex on Secretion of Gastric Juice. *Arch. Int. Med.*, 53:58, 1934.
11. Alvarez, W. C., Pilcher, F., Foley, M. A., Mayer, A. and Osterberg, A. E.: The Effects on Gastric Juice of Deprivation of Vitamin B. *Am. J. Dig. Dis. and Nutrit.*, 3:102, 1937.
12. Sure, B. and Harrelson, R. T.: Enzymic Efficiency in Avitaminosis. *Am. J. Dig. Dis. and Nutrit.*, 4:177, 1937.
13. Williams, R. R. and Spies, T. D.: Vitamin B. MacMillan, N. Y., 1938.
14. Sparks, M. I. and Collins, E. N.: The Role of Vitamin B, in the Tonus of Large Intestines. *Am. J. Dig. Dis. and Nutrit.*, 2:618, 1935.
15. Babkin, B. P.: The Physiological Factors Det. Acidity of Gastric Juice and Gastric Contents. *Canad. M. A. J.*, 29:5, 1933.
16. Chatterjee, D. D.: Intestinal Motility in Avitaminosis B and in Starved Animals. *Indian J. M. Research*, 23:191, 1935.
17. Molitor, H. and Sampson, W. L.: Quoted Annual *Rev. of Biochemistry*.
18. Elsom, K. O'Shea, Lewy, F. H. and Heublein, G. W.: Clinical Significance of Exp. Human Vitamin B Complex Deficiency. *Am. J. Med. Sci.*, 200:757, 1940.
19. Pendergrass, E. P. and Comroe, B. I.: Roentgen Study of Gastro-Intestinal Tract in Chronic Idiopathic Adult Tetany. *Am. J. Roent.*, 33:647, 1935.
20. Mackie, T. T. and Pound, R. E.: Changes in Gastro-Intestinal Tract in Deficiency States. *J. A. M. A.*, 104:518, 1935.
21. Snell, A. M. and Camp, J. D.: Chronic Idiopathic Steatorrhea, Roentgenological Observations. *Arch. Int. Med.*, 53:615, 1934.
22. Crandall, L. A., Chesley, F. F., Hansen, D. and Dunbar, J.: The Relation of P-P Factor to Gastro-Intestinal Motility. *Proc. Soc. Exper. Biol. and Med.*, 41:472, 1939.

Klin. Wochschr. 31:911-912 (1953).

The effect of inositol on basal metabolism.

by Helmut Maurer and Helmut Seckfort

Inositol, a so-called "lipotropic substance", has recently become to be discussed, especially by American authors, for the treatment of various disturbances of fat metabolism. Hereby, fatty liver and arteriosclerosis stand in the foreground. The theoretical aspects, which have led to practical, therapeutical utilization of inositol, were the subject of exhaustive experimental investigations and detailed discussions (see reviews; 1,2).

We ourselves could show in earlier tests that the intravenous injection of inositol (meso-inositol, Maizenawerke, Hamburg) caused, after a slight increase, a clear reduction of blood sugar[3,4], reduced cholesterol ester in the serum, temporarily[3-5], and practically no change in the serum protein material (determined electrophoretically)[3-6]. The question of interest to us now is whether the investigation of basal metabolism in dependence of inositol administration offers the possibility of clarifying the individual metabolism components of the altered mechanism.

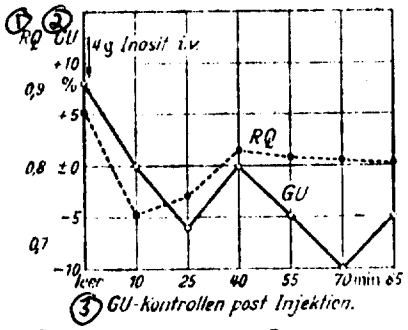


Fig. 1. Case 8, Group B.
1. Respiratory quotient
2. Basal metabolism
3. Basal metabolism - controls after injection.

Test procedure: Determinations of basal metabolism were carried out collectively on 45 test subjects who, except for 2 cases of Basedow's disease and one of endogenous emaciation, were all metabolically healthy. We performed the calorimetric tests with the Knipping apparatus, error ± 7%. After determination of the basal value, we gave, slowly intravenously, to Group a (21 cases) 2 g inositol dissolved in 20 ml 0.9% NaCl, to Group b (10 cases) 4 g inositol in 40 ml of the same solvent and to Group c (14 cases) 20 ml of the solvent alone. We performed basal metabolism determinations 10, 25, 40, 55, 70, and, in most cases, 85 minutes after the injection. Tests began on calm patients after 1 hour of absolute body rest. In most cases we determined the initial value twice. The inositol injection was endured by all patients without complications. A tabular compilation of the results is provided as by request. Case 8 from Group b is used as an example for the course of the curves (see Fig. 1).

Results: Group a. The results after injection of 2 g inositol were not uniform. In 11 of 21 cases there occurred, generally 25 minutes after the injection, a reduction of basal metabolism of 7-25% below the initial value; the lowest point was reached, on the average, after 55 minutes. While in 4 cases a slight elevation (average approximately 12%) of the basal metabolism was observed, 5 cases remained completely unchanged.

Group b. After injection of 4 g of inositol we observed in all cases a considerable reduction (around 15-25%) of the basal metabolism, which was interrupted by a small, probaly antiregulatory, limited, intermediate increase. This was clear in 6 cases and hinted at in 4 cases (see Fig. 1).

Group c. From the exclusive injection of physiological saline solution there appeared only small, irregular fluctuations, which were inside the possible error of the method. Only with 2 psychically extraordinarily unsteady female patients did the increase come to 11 and 12%.

By comparison of the respiratory quotient (RQ) there appeared the following changes: after injection of 4 g of inositol the RQ was reduced an average of about 0.071, while with 2 g an average reduction of 0.054 occurred. (No change of RQ occurred with physiological saline solution).

Discussion of results: The reduction of basal metabolism, which is obviously dependent upon the inositol dose, proceeds parallel to the already described blood sugar reduction (see above). Therefore it is presumed that the later represents the effect of a stimulation of the liver to increased glycogen formation. The present test results support this assumption in so far as pointed out by the observed reduction of the metabolism in sparing and accumulation measurements. The metabolism evidently experienced a shift in the direction of a more vagotonic assimilation phase.

The behavior of the RQ merits special observation. The observed reduction after administration of inositol indicates that an increased fat combustion occurs parallel to the blood sugar reduction. The fat evidently is taken up in increased measure to protect against the caloric need. To what extent the reduction of cholesterol ester concentration after inositol injection (see above) is herein connected is not yet known.

Summary: While after intravenous administration of inositol there occurs, as an indication of a more vagotonic assimilation phase, a decrease of basal metabolism (reduction of blood sugar), the respiratory quotient is reduced, indicating an increased fat combustion.

We are obliged to thank Maizena-Werken, Hamburg, for the friendly gift of the inositol.

Literature

[1] Weidlein: A consideration of the physiological importance of inositol. Pittsburgh: Mellon-Institute 1950. - [2] Fleury et Balatre: Les inositols. Paris: Masson & Cie. 1947. - [3] Voit u. Seckfort: Münch. med. Wschr. 1953, Nr 24, 680. - [4] Seckfort: Ärztl. Forsch. 1953. - [5] Seckfort: Ärztl. Forsch. 1953. - [6] Seckfort: Ärztl. Forsch. 1953.

Proc. Soc. Exper. Biol. & Med. 84:386-388, 1953

Effect of Inositol Feeding on Inositol Phosphatides and Other Lipids of Human Blood Plasma.* (20654)

J. M. McKIBBIN AND DAVID W. BREWER.

From the Departments of Biochemistry and Otolaryngology, State University of New York College of Medicine at Syracuse.

Concerned by the role of atherosclerosis in producing hearing loss and labyrinthine dysfunction, it occurred to one of us that a lipotropic factor might be of some clinical value. A study of such possible effects of inositol was initated and has continued for the past several years. Biochemical as well as clinical methods were included. A method has been developed for the determination of lipid inositol in various tissues including blood plasma (1). Results of the determination of the effect of inositol ingestion on the concentration of plasma lipid inositol and several other plasma lipids are summarized in this paper.

Methods. All of the patients reported in this study showed varying degrees of high tone deafness and ranged from 34 to 68 years of age. One initial blood sample was obtained before oral inositol was started. The values for the various lipid components in these samples are in the normal range for the most part and the group could not be considered lipemic. The patients then all received 1 g of inositol† 3 times daily. Blood samples were obtained at varying intervals thereafter and averaged 2 samples per patient. The duration of inositol feeding in the patients reported ranged from 1 to 12 months. None of the patients reported any untoward symptoms or reactions from the inositol. All blood samples were obtained before breakfast between 9 and 9:30 a.m. 50 ml of blood was withdrawn into a centrifuge bottle containing 2 ml of Wintrobe's oxalate. The lipid extracts of the plasma were prepared, purified, and analyzed for total lipid phosphorus and inositol by methods reported previously(1,2). Total fatty acids were determined titrametrically by a modification of the method of Stoddard and Drury(3) on the purified extract.

*Supported by a Grant from the Life Insurance Medical Research Fund.

† The inositol was generously supplied by the Corn Products Refining Co., Argo, Ill.

TABLE I. Effect of Inositol Feeding on Lipids of Human Blood Plasma in Thirteen Patients.

	Mean		Range		Mean change	S.E.	t	P
	Initial	Final	Initial	Final				
Total lipid P	295 *	310	230-389	244-376	+ 5.30 ‡	2.12	2.49	.031
Total lipid inositol	7.4*	7.3	5.5-9.4	4.5-9.8	— .8 ‡	2.82	.28	.8
Lipid inositol:phosphorus molar ratio	.025	.024	.022-.029	.019-.029	— 5.44 ‡	2.40	2.27	.04
Total cholesterol	223 †	242	160-309	183-319	+ 9.63 ‡	3.90	2.47	.033
Free cholesterol	61.9†	67.4	42.2-88.8	48.6-91.3	+ 10.0 ‡	3.60	2.78	.017
Ratio free:total cholesterol	.276	.277	.249-.291	.258-.300	+ .50 ‡	1.23	.408	.7
Ratio total cholesterol: lipid P	.757	.785	.624-.913	.689-1.078	+ 4.23 ‡	3.26	1.30	0.2
Neutral fat fatty acids	349 *	161	71-1054	13-374	—188 §	68.2	2.76	.02
Ratio neutral fat F.A.: total cholesterol	1.48	0.67	0.38-3.98	0.07-1.56	— .806§	.259	3.11	.01

* Values given in μm/100 ml plasma. † Values given in mg/100 ml plasma. ‡ Mean % change. § Mean numerical change.

Total and free cholesterol were determined on a separate aliquot of the plasma by the method of Schoenheimer and Sperry(4).

Results. A summary of the results obtained from 13 patients is given in Table I. In this summary the analyses of the last sample only after inositol feeding is chosen for comparison with the initial pre-inositol blood since it represents the maximum time of inositol intake. Cholesterol (free and total), total phospholipids and inositol phospholipids are calculated as the percent change from the initial sample. Neutral fat fatty acids are a more variable component and are therefore calculated in terms of absolute change in micromoles per cent. The statistical method for evaluation of the data is that of Fischer in which Standard Errors of the Difference are obtained using the initial value as zero. The "t" values are thus obtained as the change from the initial sample divided by the Standard Error of the Difference.

Discussion. Although the series is small it seems established that no real change occurred in the concentration of plasma inositol phosphatides incident to feeding amounts of inositol which are many times greater than the ordinary dietary intake. The ratio of inositol phosphatides to the total plasma phosphatides appears to be quite constant and all the values fall within the range of .019 to .029 in the samples reported. In another group of 6 patients who did not receive inositol and are not reported, the values also conformed to this range.

There appeared to be small but measurable mean increases in total cholesterol, free cholesterol, and total phospholipids. However, these changes are of doubtful statistical significance. These latter lipid components have been measured in several inositol feeding experiments on man and animals by others who reported (a) decreases in total cholesterol (5-8) and phospholipids(7) or (b) little or no change in cholesterol(9-12), phospholipids (10,12) and low density lipoproteins(12). In cholesterol fed rabbits, inositol was reported to be both ineffective(13) and effective(14) in preventing the hypercholesteremia. In old hens inositol reduced cholesteremia(15) but failed in combination with choline to reduce the cholesteremia and general lipemia in chicks fed cholesterol(16) or treated with estrogen (17). It seems clear that no simple relationship, if any, exists between exogenous inositol and blood plasma total cholesterol, free cholesterol or total phospholipids.

In this study, the neutral fat fatty acids decreased with inositol feeding, and more strikingly in the more lipemic patients—*i.e.*, those with initial cholesterol values over 200 mg%. The decreases as recorded appear to be significant. However, caution must be exercised in the interpretation of changes in as highly variable a fraction as the neutral fat. The mean decrease in the "lipemic" group is less than the magnitude of what must be considered a "normal" range for neutral fat. Moreover, no consistant decrease in the neutral fat fraction was observed in cholesterol fed chicks(15) or estrogen treated chicks(16) given inositol with choline. Nevertheless,

these data are of considerable interest since at least a part of this fraction is associated with low density lipoproteins(18) which in turn are thought by some to be associated with the development of atherosclerosis. Studies are in progress to further examine such a relationship between inositol and neutral fat.

Summary. A group of 13 patients with high tone deafness was treated with 3 g oral inositol daily for periods of 1 to 12 months. No change resulted in the fasting plasma inositol phosphatide concentration; there were minor increases in total and free cholesterol and total phospholipids, and there was an apparent decrease in neutral fat.

1. Taylor, W. E., and McKibbin, J. M., *J. Biol. Chem.*, 1953, v201, 609.

2. ———, *ibid.*, 1951, v188, 677.

3. Stoddard, J. L., and Drury, P. E., *ibid.*, 1929, v84, 741.

4. Schoenheimer, R., and Sperry, W. M., *ibid.*, 1934, v106, 745.

5. Herrmann, G. R., *Exp. Med. and Surg.*, 1947, v5, 149.

6. Felch, W. C., and Dotti, L. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 376.

7. Felch, W. C., Keating, J. H., and Dotti, L. B, *Am. Heart J.*, 1952, v44, 390.

8. Sherber, D. A., and Levites, M. M., *J. A. M. A.*, 1953, v152, 682.

9. Lupton, A. M., Battafarano, T. W., Murphy, F. E., and Brown, C. L., *Ann. Western Med. and Surg.*, 1949, v3, 342.

10. Leinwand, I., and Moore, D. H., *Am. Heart. J.*, 1949, v38, 467.

11. Popkin, R. J., *Angiology*, 1951, v2, 398.

12. DeWind, L. T., Michaels, G. D., and Kinsell, L. W., *Ann. Int. Med.*, 1952, v37, 344.

13. Moses, C., Rhodes, G. L., and Delacio, A, *Angiology*, 1952, v3, 238.

14. Dotti, L. B., Felch, W. C., and Ilka, S. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 167.

15. Herrmann, G. R., *ibid.*, 1946, v63, 436.

16. Stamler, J., Bolene, C., Harris, R., and Katz, L. N., *Circulation*, 1950, v2, 714.

17. ———, *ibid.*, 1950, v2, 722.

18. Jones, H. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P., Graham, D. M., Strisower, B., and Nichols, A. V., *Am. J. Med.*, 1951, v11, 358.

Received September 28, 1953, P.S.E.B.M., 1953, v84.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 80, (1959)

The Metabolism in the Rat of Photosynthetically Prepared *Myo*-Inositol-C^{14} [1]

Ezio A. Moscatelli[2] and Joseph Larner[3]

From the Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois

Received March 17, 1958

Introduction

In the course of investigating the function of *myo*-inositol in animal systems it has been thought desirable to elucidate the pathways of carbon in *myo*-inositol biosynthesis and degradation. Earlier work by Daughaday, Larner, and Hartnett (1) and Halliday and Anderson (2) has demonstrated the conversion of C^{14}-labeled glucose to *myo*-inositol-C^{14} in the rat. Stetten and Stetten (3) have demonstrated the conversion of uniformly deuterium-labeled *myo*-inositol to urinary glucose in phlorizinized rats. Subsequent work by Posternak (4), using *myo*-inositol uniquely deuterated in the 2-position, confirmed this and further showed that the observed conversion of *myo*-inositol to glucose could not proceed as a simple, one-step ring opening.

Hokin and Hokin (5) recently reported the incorporation of *myo*-inositol-2-H^3 into pigeon pancreas and brain cortex phospholipides, while Agranoff (6) noted incorporation of tritium into the lipides of rat liver, kidney, lung, brain, and pancreas following intraperitoneal injection of *myo*-inositol-2-H^3. This compound was also used by Agranoff *et al.* (7) to study the enzymic route of inositol incorporation into lipide in guinea pig kidney mitochondrial preparations.

The present paper describes the photosynthetic preparation of *myo*-inositol-C^{14} and the use of this compound to quantitatively measure *myo*-

[1] This work has been supported in part by a grant from the Lipotropic Foundation.

[2] Predoctoral Research Fellow of the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service. The work reported here is to be part of a thesis to be submitted by Ezio A. Moscatelli in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Ill.

[3] Present address: Department of Pharmacology, Western Reserve University, Cleveland, Ohio.

inositol dissimilation along three pathways in the intact, normal rat.[4] These are (*a*) oxidation to CO_2; (*b*) incorporation into lipides of brain, liver, kidney, and heart; and (*c*) conversion to liver glycogen.

Experimental

1. *Photosynthetic Preparation of* myo-*Inositol-*C^{14}

Single cross hybrid seed corn[5] (WF9xM14) was germinated in wet vermiculite[6] in a 9 × 14 in. Pyrex baking dish. Corn seeds were scattered approximately 1 in. apart on a one half inch layer of vermiculite and covered with another half inch of this material. Tap water was added to a level just below the seeds. The dish was wrapped in brown paper and placed in a greenhouse. After 2–3 days the brown paper was removed and the plants were allowed to grow under normal greenhouse conditions. The vermiculite was kept moist another 5–7 days. Eight- to 10-day-old plants, after overnight starvation in the dark, were placed in a photosynthesis chamber in a small polyethylene pot containing moist vermiculite. A vial containing $BaC^{14}O_3$ was suspended next to a rubber plug through which a hypodermic needle could be thrust into the vial. The chamber was partially evacuated, then $C^{14}O_2$ was released by adding 35% perchloric acid to the $BaC^{14}O_3$, using a hypodermic syringe. Atmospheric pressure was restored by the admission of air, and the plants were allowed to grow in the presence of light furnished by six 17-in., 15-w. cool-white fluorescent light tubes placed 6–8 in. from the plants. Red light was furnished by two turns of a helical high pressure neon tube 5 in. from the plants.

The rate of disappearance of radioactivity from the air in the chamber was determined externally by the use of an analytical count rate meter.[7]

The Geiger-Müller tube was fixed approximately 2 cm. above a thin mica window which was joined to the chamber by a female 18/23 joint. By this means, it was observed that more than 90% of the radioactive doses used were taken up in 24 hr. After various time periods, the plant tissues (leaves and/or roots) were minced with scissors, and both free and bound inositol were extracted by stirring 15 min. with each of the following solvents (20 ml./g. tissue): three times with hot water (containing 5% ethanol to more effectively wet the leaves); twice with hot 50% aqueous ethanol; and once each with ethanol, 50:50 ethanol-ether, and ether.

The combined extracts were concentrated *in vacuo* to a sirup and hydrolyzed by refluxing overnight in 6 *N* sulfuric acid. One hundred milligrams carrier *myo*-inositol was added, and the *myo*-inositol-C^{14} was isolated essentially by the method of Halliday and Anderson (2), with the exception that Crystalab Deeminite L-10,[8] a mixed ion-exchange resin, was used rather than Dowex 50 and IRA-400. The *myo*-inositol-C^{14} was crystallized out of aqueous solution by the addition of ethanol and storing at low temperature, and was recrystallized to constant specific activity from water-ethanol. Four to five recrystallizations were generally required. In a typical preparation: m.p. of isolated compound = 222–226°C.; m.p. of hexaacetate = 217–218°.

Paper chromatography of the isolated compound using 80% aqueous ethanol revealed a single, radioactive spot migrating at the same R_f as authentic *myo*-inositol. Infrared absorption and x-ray diffraction data confirmed the identity and purity of the compound.[9]

[4] A preliminary report of this work has been presented to the Federation of American Societies for Experimental Biology at Chicago, Illinois, in March, 1957 (8).

[5] *Myo*-Inositol-C^{14} has also been isolated in this laboratory from radioactive leaves and stems of *Hevea brasiliensis*, which was kindly supplied by J. Skok of the Argonne National Laboratories.

[6] Zonolite Company, Chicago, Ill.

[7] Analytical Count Rate Meter 1620, Nuclear Instruments Corp., Chicago, Ill.

[8] Crystal Research Laboratories, Hartford, Conn.

2. *Technique of Animal Experiments*

Photosynthetically prepared *myo*-inositol-C^{14} was administered to rats by intraperitoneal injection. Rats were kept in closed glass metabolism chambers under a slow stream of CO_2-free air (6 l./hr.), such that expired CO_2 was collected in sodium hydroxide scrubbing towers. The alternate use of parallel scrubbing towers made possible the continuous collection of all the $C^{14}O_2$ expired. CO_2 was collected for 10–12½ hr. after the initial *myo*-inositol injection, after which time rats were killed by decapitation.

3. *Glycogen Isolation*

The liver glycogen was isolated essentially by the method described by Stetten, Katzen, and Stetten (9). After 3–4 ethanol reprecipitations from aqueous solution, the glycogen was further purified by exhaustive dialysis in the cold against distilled water.

4. *Isolation of Total Purified Lipides*

The total purified lipides from each of four organs, liver, kidney, brain, and heart, were prepared by homogenizing each tissue 2–3 min. in a Waring blendor with chloroform–methanol (2:1), using approximately 50 ml. solvent/g. tissue, then purifying the extract by the water wash method of Folch *et al.* (10). The efficiency of the Folch water wash procedure was checked by adding *myo*-inositol-C^{14} to lipide extracts in control experiments, in which it was found that at least 90% of the water-soluble radioactivity could be removed in three washes. After four such washes, the purified lipide preparations were examined paper chromatographically for the presence of free *myo*-inositol, using the lutidine–methanol–acetic acid (16:4:1) solvent system of Rouser *et al.* (11). Chromatograms were developed by a modification of the method of Trevelyan *et al.* (12), by dipping first in an acetonic solution of silver nitrate,[10] then in 0.5 *N* sodium hydroxide in 90% aqueous methanol. Essentially no silver nitrate-reacting material was found in any of the preparations, under conditions in which added free *myo*-inositol could easily be demonstrated in controls.

The bulk of the lipide and radioactivity migrated to the upper third of the paper under these conditions, whereas it could be shown that added free *myo*-inositol remained essentially at the origin.

The water-washed total lipides were therefore further subjected to paper chromatographic analyses, and were exhaustively extracted with chloroform–methanol (2:1) from the chromatograms for combustion.

[9] We are indebted to J. J. Brader and J. Thomas of this Department for carrying out infrared and x-ray analyses, respectively.

[10] This solution was prepared by adding 10 vol. water to 1 vol. of saturated aqueous silver nitrate, then adding 200 vol. acetone.

TABLE I

Photosynthetic Preparation of myo-*Inositol-*C^{14} *from* $C^{14}O_2$ *in Corn Plants*

Preparation No.	C^{14} administered[a]	Photosynthetic period	Tissue used for isolation		C^{14} yield as *myo*-inositol-C
	d.p.m.	*hr.*		*g.*	%
1.[b](*a*)	4.5×10^9	25	Leaves	5.9	0.051
(*b*)		57	Leaves	9.1	0.068
(*c*)		80	Roots	12.6	0.020
2.	21.3×10^9	51	Leaves and roots	29.6	0.065

[a] As $C^{14}O_2$ released from $BaC^{14}O_3$ by the action of perchloric acid.

[b] Part of the leaves were removed after 25 hr., the remainder allowed to grow for 57 hr. The combined roots were used after 80 hr.

5. *Analytical Methods*

Radioactivity was measured as $C^{14}O_2$ with a vibrating-reed electrometer model 30.[11] Respiratory CO_2 was released with 35% perchloric acid, while other materials were oxidized to CO_2 essentially by the wet combustion procedure of Van Slyke *et al.* (13). In order to minimize the formation of I_2, much lower KIO_3 to $K_2Cr_2O_7$ ratios were used in the combustion mixture. Since in the procedure used total $C^{14}O_2$ from each sample is counted directly in a chamber, the formation of small amounts of $C^{14}O$ could not affect the radioactivity measurements.

The identity and purity of isolated liver glycogen was established by quantitative determination of glucose, after acid hydrolysis, by two independent methods. Reducing power was determined by the method of Nelson (14) and by spectrophotometric enzymic assay with hexokinase and glucose-6-phosphate dehydrogenase (15).

Results

1. *Photosynthetic Preparation of* myo-*Inositol-*C^{14}

As may be calculated from the data of Table I, only a small difference in per cent incorporation per weight of tissue was observed between the 25- and 57-hr. photosynthetic periods. A smaller per cent incorporation was found in the roots than in the leaves in an 80-hr. period.

The isolation of *myo*-inositol-C^{14} reported here represents proof that young corn plants can incorporate C^{14} from CO_2 into *myo*-inositol while growing in the presence of light. Since there are no reactions by which a one-carbon unit can exchange into *myo*-inositol, this evidence strongly indicates a biosynthesis. Since radioactive *myo*-inositol was found in both leaves and roots, no conclusion can be drawn about either the site or the mechanism of synthesis. Darbre and Norris (16) have recently demonstrated that in oats germinated in the dark there is a decrease in bound forms of *myo*-inositol only partially compensated by an increase in free

[11] Applied Physics Corp., Pasadena, Calif.

myo-inositol. Richardson and Axelrod (17) have reported similar findings and in addition have shown that the 10-day-old etiolated pea seedling as well as the immature pea pod can incorporate $C^{14}O_2$ into free inositol in the presence of light.

It is of interest to note that the over-all radioactive yield as *myo*-inositol-C^{14} of *ca.* 0.07% is in the same range as the 0.055–0.076% radioactive yields reported by Halliday and Anderson (2) for the conversion in the rat of glucose-1-C^{14} to *myo*-inositol-C^{14}. Daughaday, Larner, and Hartnett (1), using uniformly labeled glucose C^{14}, obtained yields of about 0.01%.

A chemical synthesis of *myo*-inositol-C^{14} in a yield of 2.2% has been described by Weygand and Schulze (18).

2. *Animal Experiments*

The metabolic data reported here are derived from three separate experiments, the differences among which will be described in the course of reporting the data below.

(*a*) *Oxidation of* myo-*Inositol-*C^{14} *to* $C^{14}O_2$. The results of $C^{14}O_2$ collection in Expt. No. 1, in which a fasted, 85 g. female rat was used, are presented in Fig. 1. Two hundred milligrams of D-glucose was initially injected intraperitoneally to stimulate the formation of liver glycogen, followed, after 45 min., by an intraperitoneal injection of 897,000 disintegrations/min. (d.p.m.) of *myo*-inositol-C^{14} with a specific activity of 41,500 d.p.m./mg. Six hours after the first injection, another 1,015,000 d.p.m. of the above *myo*-inositol-C^{14} was injected into the animal. $C^{14}O_2$ radioactivity collected represented 25.7% of the total radioactive dose injected. In Expts. 2 and 3 (see below) using *myo*-inositol-C^{14} which had in one case approximately half, in the other case approximately three times the specific activity of the preparation used in Expt. 1, total radioactive yields of $C^{14}O_2$ were obtained which were very similar to the one shown in Fig. 1. As indicated by the data in Table II, the vigorous oxidation observed was larger in the fasted animals than in the fed one, the relative yield being more than twice as great. When calculated in terms of amounts of *myo*-inositol oxidized assuming random labeling, the values obtained are 12, 18, and 3 mg. for Expts. 1, 2, and 3, respectively.[12]

(*b*) *Incorporation of* myo-*Inositol-*C^{14} *into Total Lipides.* The data reported in Table III are from Expt. 3, which was performed using a rat previously maintained for 7 days on a high protein, high carbohydrate diet[13]

[12] These values are uncorrected for dilution by endogenous *myo*-inositol. Calculation of extracellular dilution assuming a blood level of 3 mg. % (19) and extracellular volume of distribution of 40% leads to a negligible correction.

[13] The diet used simulated that described by Munro and Naismith (20), and consisted of 78 parts by weight of ground Purina Laboratory Chow plus 11 parts each of sucrose and Labco Vitamin-free casein.

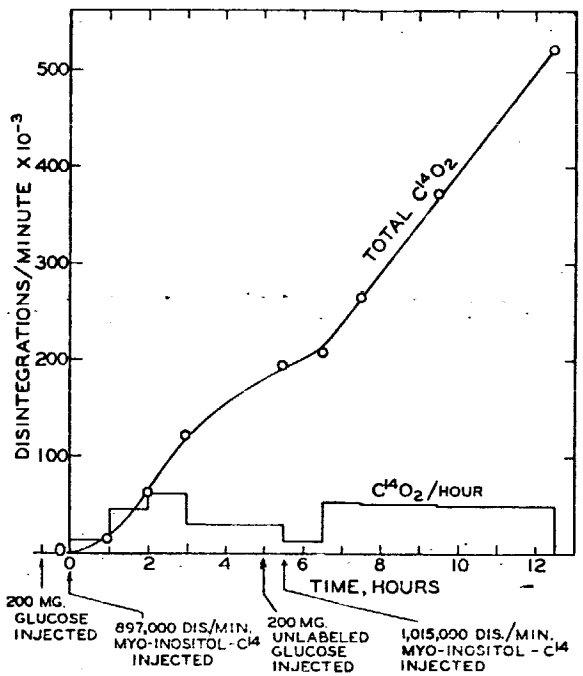


FIG. 1. $C^{14}O_2$ expiration after intraperitoneal injection into a fasted rat of uniformly labeled *myo*-inositol-C^{14}. $C^{14}O_2$ collected at each of the indicated time periods is given by the area under each horizontal line. The upper curve shows the cumulative radioactivity in disintegrations/min. $\times 10^{-3}$ in the expired $C^{14}O_2$.

TABLE II

Total Yields of $C^{14}O_2$ from myo-*Inositol-C^{14} Administered to Rats*

Experiment No.	*myo*-Inositol-C^{14} administered		$C^{14}O_2$ expired	
	Radioactive dose	Specific activity	Total yield	Relative yield
	d.p.m.	*d.p.m./mg.*	*d.p.m.*	%
1	1,912,000	41,500	520,000	25.7
2	2,090,000	25,100	465,000	22.3
3	4,200,000	124,000	420,400	10.1

designed to insure large amounts and high turnover rates of tissue phospholipides, as observed by Wikramanayake *et al.* (21).

The rat used in Expt. 3, a 130-g. female, was intraperitoneally injected at 0, 2, 4, and 6 hr. with 1 ml. of 500 mg./ml. aqueous solution of D-glucose during the 10-hr. experiment, these injections being given simultaneously with intraperitoneal injections of *myo*-inositol-C^{14}. The amount of radio-

TABLE III

Incorporation of Radioactivity from myo-*Inositol-C^{14} into Total Purified Lipides of Rat Organs*[a]

Organ	Total purified lipides		Radioactivity of purified lipides		
			Total activity	Specific activity	Incorporation
	g.	*mg.*	*d.p.m.*	*d.p.m./mg.*	%
Brain	1.2	108	2,730	25.3	0.07
Liver	4.7	225	13,280	59.0	0.32
Kidney	1.4	119	8,350	70.1	0.20
Heart	0.5	19	3,870	204.0	0.09

[a] These data are from Expt. 3, using a rat previously maintained 7 days on a high protein, high carbohydrate diet.

TABLE IV

Conversion of myo-*Inositol-C^{14} to Liver Glycogen in the Rat*

Experiment No.	Liver glycogen	Liver glycogen radioactivity		
		Total activity	Specific activity	Radioactive yield
	mg.	*d.p.m.*	*d.p.m./mg.*	%
1		2,470		0.12
2	7.8[a]	3,200	410	0.16
3[b]	81.0	123,000	1,520	3.16

[a] Determined as glucose, by reducing power and microenzymically with hexokinase and glucose-6-phosphate dehydrogenase.

[b] Starved rats were used in Expts. 1 and 2, whereas in Expt. 3, the animal used had been previously maintained 7 days on a high carbohydrate, high protein diet.

activity available to the rat in this experiments was 4,200,000 d.p.m.[14] of *myo*-inositol-C^{14} with a specific activity of 124,000 d.p.m./mg. In Expts. 1 and 2, where starved rats were used, considerably lower incorporations into lipide were observed and were of the order of 10% of the incorporations observed in Expt. 3.

(*c*) *Conversion of* myo-*Inositol-C^{14} to Liver Glycogen.* The incorporation of radioactivity into liver glycogen is summarized in Table IV. Experiment 2 was conducted in a manner similar to No. 1, except that in this case there was administered a total of 2,090,000 d.p.m. of *myo*-inositol-C^{14} with a specific activity of 25,100 d.p.m./mg. The rat used was a 24-hr. fasted, 90-g. female. In both Expts. 1 and 2, the data in Table IV indicate similar, low

[14] The total radioactivity injected was 5,320,000 d.p.m. of which 1,120,000 d.p.m. was present in the peritoneal fluid at the end of the experiment. The accumulation of fluid was observed only in this experiment and was due to the repeated injections of hypertonic glucose.

radioactive yields. In Expt. 3, where the high carbohydrate, high protein diet-fed animal was used, the radioactive yield was increased approximately 20-fold.

(*d*) *Recoveries of Radioactivity.* In Expt. 3, in which all three pathways were investigated quantitatively, the total recovery in these routes of the administered radioactive dose was 13.9%. Higher recoveries of approximately 25%, represented almost entirely by $C^{14}O_2$, were noted in the experiments in which starved animals were used.

Recoveries of radioactivity in the urine were 22.8, 56.0, and 6.2% in Expts. 1, 2, and 3, respectively. Almost 50% of the administered radioactive dose was found in the carcass of the rat in Expt. 3. In this experiment an over-all recovery of approximately 66% of administered radioactive dose was obtained.

Discussion

The presence of free *myo*-inositol in rat blood at a level of approximately 3 mg.% has previously been demonstrated (19), and it has been known for some time that one pathway of its metabolism in the rat is conversion to glucose, as found in the urine of phlorizinized animals, in the order of a 6 or 7% conversion (3, 4). In the present work it has been shown that *myo*-inositol-C^{14} is rapidly metabolized in the intact rat, as demonstrated by a vigorous oxidative dissimilation to $C^{14}O_2$. The high conversion noted in these experiments, especially in fasted animals, is compatible with the recent demonstration of the formation of DL-glucuronic acid from *myo*-inositol by kidney extracts (22). Variable yields of $C^{14}O_2$ may provisionally be explained on the basis of the recent findings of Eisenberg (23) on the variable $C^{14}O_2$ yields from glucuronate-C^{14} in the rat.

The work of Posternak (4) and Stetten and Stetten (3), which established the conversion of *myo*-inositol to glucose, has been here confirmed using a carbon-14 label. Labeling of liver glycogen was used as a device for demonstrating the formation of D-glucose-C^{14}. Stetten and Stetten (3) attempted to demonstrate glycogen labeling in their deuterium-label experiments, but were unable to isolate any glycogen from their fasted rats. In the present experiments this difficulty was obviated by administration of glucose before each dose of *myo*-inositol-C^{14}.

It has been noted that in the well-fed animal of Expt. 3 the radioactive yield as liver glycogen was increased approximately 20-fold. A possible explanation for this is that the over-all conversion of *myo*-inositol to glucose is an endergonic process.

In confirmation of the work of Agranoff (6), incorporation into lipide has been demonstrated to be yet a third pathway of *myo*-inositol metabolism in the intact rat. The largest total incorporations were found in the

lipides from liver and kidney. It is interesting to note the wide variations in specific activities of the lipides from the various organs, apparently reflecting considerable differences in turnover rates of the *myo*-inositol components.

Acknowledgment

The authors wish to thank Mrs. C. M. McNickle for her valuable technical assistance.

Summary

Three pathways of *myo*-inositol metabolism in the whole rat were quantitatively investigated using photosynthetically prepared *myo*-inositol-C^{14}.

A small conversion to glucose is shown by liver glycogen labeling. Comparable amounts are incorporated into organ lipides. A much larger conversion is represented by a vigorous oxidative dissimilation to CO_2.

References

1. Daughaday, W. H., Larner, J., and Hartnett, C., *J. Biol. Chem.* **212**, 869 (1955).
2. Halliday, J. W., and Anderson, L., *J. Biol. Chem.* **217**, 797 (1955).
3. Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.* **164**, 85 (1946).
4. Posternak, T., Schopfer, W. H., and Reymond, D., *Helv. Chim. Acta* **38**, 1283 (1955).
5. Hokin, L. E., and Hokin, M., *Federation Proc.* **16**, 198 (1957).
6. Agranoff, B. W., *Federation Proc.* **16**, 379 (1957).
7. Agranoff, B. W., Bradley, R. M., and Brady, R. O., *Biochim. et Biophys. Acta* **25**, 445 (1957).
8. Moscatelli, E. A., and Larner, J., *Federation Proc.* **16**, 223 (1957).
9. Stetten, M. R., Katzen, H. M., and Stetten, D., Jr., *J. Biol. Chem.* **222**, 587 (1956).
10. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N., *J. Biol. Chem.* **191**, 833 (1951).
11. Rouser, G., Marinetti, G. V., Witter, R. F., Berry, J. F., and Stotz, E., *J. Biol. Chem.* **223**, 485 (1956).
12. Trevelyan, W. E., Procter, D. P., and Harrison, J. S., *Nature* **166**, 444 (1950).
13. Van Slyke, D. D., Plazin, J., and Weisiger, J. R., *J. Biol. Chem.* **191**, 299 (1951).
14. Nelson, N., *J. Biol. Chem.* **153**, 375 (1944).
15. Slein, M. W., *J. Biol. Chem.* **186**, 753 (1950).
16. Darbre, A., and Norris, F. W., *Biochem. J.* **64**, 441 (1956).
17. Richardson, K. E., and Axelrod, B., *Plant Physiol.* **32**, 334 (1957).
18. Weygand, F., and Schulze, E., *Z. Naturforsch.* **11b**, 370 (1956).
19. Daughaday, W. H., and Larner, J., *J. Clin. Invest.* **33**, 1075 (1954).
20. Munro, H. N., and Naismith, D. J., *Biochem. J.* **54**, 191 (1953).
21. Wikramanayake, T. W., Munro, H. N., Naismith, D. J., and Hutchison, W. C., *Biochem. J.* **55**, 640 (1953).
22. Charalampous, F., and Lyras, C., *Federation Proc.* **16**, 164 (1957).
23. Eisenberg, F., Jr., *Federation Proc.* **16**, 176 (1957).

Natsume, K.: STUDIES ON MYOINOSITOL. (III) EFFECT OF AN EXCESS DOSAGE OF MYOINOSITOL ON THE PATTERN OF LIPIDS IN THE LIVER OF YOUNG RATS. Bitamin, Vol. 32, No. 4, pp. 363-8, 1965. Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya.

Myoinositol exerts favorable effects on the growth of higher animals such as mice (1,2) and white rats (3) as well as microorganisms. However, the knowledge on inositol deficiency in higher animals is extremely limited. Besides alopecia and spectacled eye (4) in mice, the only other manifestation of inositol deficiency so far observed experimentally is the increased mortality among female hamsters at parturition (5). The lack of clear definition of the deficiency symptom has been a great handicap in the study of its growth stimulation effect, and as a result, its effect on higher animals as a vitamin has never been fully clarified.

While the ability of higher animals to biosynthetize myoinositol (6-9) is being substantiated in recent years, it has also been found that certain proliferative tissues in higher animals require myoinositol for their growth (10,11). Attention has also been drawn to the role of free inositol in cells (12-14) and the action mechanism of inositol phospholipid (15,16).

This study was designed to evaluate the nutritional value of myoinositol and this paper discusses the affinity of myoinositol toward lipids as reflected in the hepatic lipid composition in young white rats following the consecutive administration of the compound.

EXPERIMENTAL MATERIALS AND PROCEDURE

(1) EXPERIMENTAL ANIMALS AND GROWTH EXPERIMENT

Approximately 20 days old male Wistar white rats weighing approximately 50 g were employed for the growth experiment, and 3 month old rats of the same species weighing approximately 120 g, for the comparative experiment. The experiments followed the procedures described in the previous paper. The feed was prepared according to the description by Forker et al. (17), except for the addition of myoinositol. The cage temperature was controlled within the range of 18 - 23°C.

(2) ADMINISTRATION OF MYOINOSITOL

As shown in Table 1, 0.5 ml of 0.1, 1, 2, and 10% aqueous myoinositol solutions were compulsorily administered daily to the animals of groups B, C, D, and E, respectively, by the oral route. Since myoinositol at a concentration of 10% does not dissolve at normal temperature, the compound at that concentration was given in the form of a suspension. In view of a possibility that the compulsory administration of 0.5 ml water may exert an effect on the animals, 0.5 ml of distilled water was also forced on the animals of group A (control group).

(3) EXTRACTION OF HEPATIC LIPIDS

The extraction followed Entenman's procedure (18). The white rats were clubbed to death, and the blood was drawn by severing the carotid artery. The abdomen was bisected, and the liver was removed and weighed. The blood drawn from the carotid artery was used for the determination of serum cholesterol. The liver was sliced, immersed in an approximately

5-fold quantity of an ethanol-diethylether mixed solution (at 3 : 1, v/v), and pulverized with an Ultra-turrax. A part of the pulverization solution was dried for 24 hours in a drier at 110°C, and the dry weight of the liver was calculated. The rest of the pulverized organ was combined with 50 ml of a solvent, and extracted for 1 hour in a warm bath at 60°C. The extraction was repeated by adding the same volume of solvent to the residue after each centrifugal separation. After repeating extraction three times, the residues were washed with ether, and the extract solutions and the wash solutions were combined. The combined extract solutions were condensed by heating until dryness, then dissolved in petroleum ether, and transferred to a measuring flask to be used for the analysis of liver lipids.

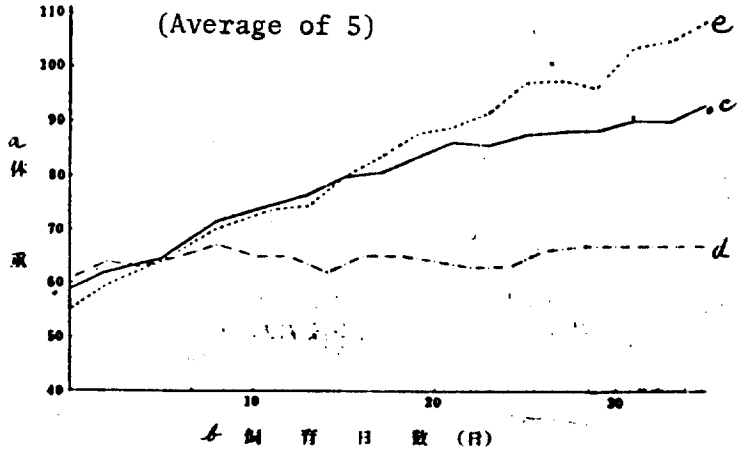


FIGURE 1. The Effect of Myoinositol on the Growth of Young White Rats

Keys: a, body weight; b, days; c, without myoinositol; d, myoinositol at 50 mg/day; e, myoinositol, at 0.5 mg/day.

TABLE 2. THE EFFECT OF ORAL MYOINOSITOL ON THE URINE MYOINOSITOL LEVEL

a ミオイノシトール投与量(mg/頭/日)	0	0.5	5	10	50
b 24時間尿中ミオイノシトール(mg)	0.182	0.155	0.256	0.140	0.175

c 各群3頭の算術平均値

Keys: a, dose (mg/animal/day); b, myoinositol in 24-hr urine (mg); c, arithmetic average of 3 animals.

TABLE 3. THE EFFECT OF MYOINOSITOL ON 3-MONTH OLD WHITE RATS

a ミオイノシトール投与量(g/kg/日)	b 実験開始前体重(g)	c 1ヵ月後体重(g)	d 体重増加(g)	e ネズミの外観
0	115	176	61	特別な変化なし f
0.005	110	165	55	特別な変化なし f
0.05	114	180	66	特別な変化なし f
0.5	116	152	36	体重増加は小さいが特別な所見はない f
5	114	180	66	特別な変化なし f

Arithmetic average of 5 animals.

Keys: a, dose (g/kg/day); b, initial body weight (g); body weight at 1 months later; d, weight gained (g); external appearence of rat; f, normal; small weight gain, but otherwise normal

(4) LIPID ANALYSIS

For the determination of the total lipids in the liver, the petroleum ether was distilled out from a part of the above specimen, and the residue was dried for 24 hours at room temperature in a vacuum desiccator over phosphorus pentoxide, and weighed.

For the determination of the P concentration in the lipids, the phosphorus was transformed into inorganic phosphorus according to Allen's wet calcination method (19) and the specimen was subjected to colorimetric determination according to Nakamura's modified procedure (20).

The N concentration in the lipids was determined by the normal procedure, i.e., after the decomposition with concentrated sulfuric acid, the produced ammonium was determined by colorimetry according to the indophenol method (21).

The total cholesterol level in the liver and the serum were determined by Zlatkis' colorimetric procedure (22).

(5) URINE INOSITOL ANALYSIS E

The inositol discharged into the urine was measured by the chemical quantitative determination procedure for the inositol found in a large quantity in sugars as described in the previous report (23). First of all, the white rat was placed in a cage for urine collection and a 24-hour portion of urine was collected. The cage was washed with 100 ml of distilled water, the wash solution and the urine were combined, and the mixture was treated by means of a Dowex-50 (H'-type) column measuring 1.5 cm in inner diameter and 7 cm in height, and a Dowex-1 (OH-type) column measuring 1.5 cm in inner diameter and 7 cm in height, in order to remove ionic materials by adsorption. Subsequently, sugars were removed by oxidation. The urine (approximately 120 ml) thus chlorinated was combined with 5 ml of 0.3 N $Ba(OH)_2$, heated for 10 minutes in boiling water, and, after cooling, combined with 5 ml of 5% $ZnSO_4$. The precipitates were separated with a centrifuge. The precipitates were washed with 10 ml of distilled water twice, and the wash solutions were combined with the supernatants. The mixture was treated with a Dowex-50 (H-type) column and a dowex-1 (OH-type) column, concentrated in a warm bath at 50°C until the quantity was 2 - 4 ml under reduced pressure. Distilled water was added to the solution until the total quantity was 5 ml, and the mixture was used as the sample solution for the determination of inositol. A 2 ml portion of the solution was subjected to the periodic acid oxidation procedure for the determination of inositol. The quantitative determination by the anthrone method (24) was carried out before and after the removal of sugars by oxidation in order to confirm the elimination of sugars.

EXPERIMENTAL RESULTS

The consecutive administration of 0.5 - 5 mg of myoinositol exerted a favorable effect on the growth of young white rats, but its large dose administration (50 mg/animal/day) did not necessarily contribute to the growth of animals. Figure 1 illustrates the overall growth of the control group, the group treated with standard dose, and the group treated with a large dose of myoinositol in terms of weight gain. The large dose group indicates slight growth retardation from the 10th - 15th day after the onset of administration. What was

particularly notable in the external appearence was the staining of the hair due to excessivefatty secretion, loss of appetite, and subsequent general hyposthenia in 4 out of the 24 cases which were given myoinositol daily at a daily dosage of 50 mg. These symptoms showed no reproducibility, and cannot be directly attributed to the large dose of myoinositol. However, the drug showed a tendency to slightly retard the growth at a large dosage at a significant rate of reproducibility.

As noted in Table 2, the amount of myoinositol excreted into the urine showed no significant difference between the groups. This signifies that, once myoinositol is introduced internally, it is always metabolized into some form. The substance may be directly excreted into the feces, but since the analytical procedure used in this experiment is not expected to provide reliable measured values, this aspect will be pursued in the future.

TABLE 1. DOSAGE OF MYOINOSITOL

a	ミオイノシトール水溶液濃度 (%) b	投与液量 (ml) c	ミオイノシトール量 (mg/頭/日) d
A群	0(蒸留水) e	0.5	0
B群	0.1	0.5	0.5
C群	1	0.5	5
D群	2	0.5	10
E群	10	0.5	50

Keys: a, group; b, concentration of myoinositol in aqueous solution (%); c, amount of solution administered (ml); d, dosage of myoinositol (mg/animal/day); e, distilled water

The growth inhibitive effect as observed in white rats is important with regard to the determination of the limit of dosage in the event that the compound is administered for a therapeutic purpose such as for the treatment of fatty liver or arteriosclerosis. In the present investigation, a large dose of myoinositol was administered consecutively to 3 months old white rats (weighing about 120 g) and changes were observed. As shown in Table 3, however, the large dose administration exerted no effect on the condition of growth.

This outcome seems to indcate that the dosage limit of this compound is considerably high from the standpoint of its toxicity. However, since the large dose administration to young white rats inhibited their growth, it is not at all unreasonable to expect a difference in its biological behavior between young white rats in the stage of active growth and mature rats. It is also deemed natural to attribute the difference in biological behavior to the difference in the growth capacity of the two groups, in other words, the difference in the cell multiplication speed.

After raising the young white rats (20 days old) with the feeds containing myoinositol at the dosages shown in Table 1 for 45 days, the liver lipid patterns of these groups were compared. The results are shown in Table 4. The experiment revealed that the administration of myoinositol caused no significant fluctuation of the total lipid and cholesterol levels in the liver, as long as the breeding condition was normal, i.e., without a cause of fatty liver or atheroma. Furthermore, as Table 5 demonstrates, no significant fluctuation was shown in the serum total chlesterol level. However, the P and N in the liver lipids

exhibited fluctuations. More specifically, as shown in Table 6, the liver P/N ratio increased with higher dosage of myoinositol. If the P level in the total lipid is assumed to reflect the total phospholipid level, and the N, the concentration of nitrogen-containing phospholipids such as choline phospholipid and sphingomyelin, the above results indicate that the increase in dosage elevates liver phospholipids. Conversely, however, the nitrogen-containing phospholipids exhibited a relatively downward trend.

However, there has been no experimental result indicating that the effect of this compound on the composition of liver lipid composition is directly related to the growth retardation of white rats.

The microscopic examination of hematoxylin-eosin preparations of the organs from the white rats of different groups revealed no significant difference between the groups.

TABLE 4. THE EFFECT OF MYOINOSITOL ON THE LIVER TOTAL LIPID AND TOTAL CHOLESTEROL LEVELS

a ミオイノシトール投与量 (mg/頭/日)	b シリーズI		c シリーズII		d シリーズIII	
	e 総脂質 (g)	コレステロール f (mg)	総脂質 e (g)	コレステロール f (mg)	e 総脂質 (g)	コレステロール f (mg)
0	0.176	15.4	0.145	10.4	0.164	18.0
0.5	0.207	14.4	0.151	8.4	0.160	17.4
5	0.184	15.9	0.137	11.8	0.163	15.8
10	0.169	14.0	0.128	11.6	0.148	14.5
50	0.180	15.9	0.145	14.6	0.137	15.1

The total lipid and cholesterol are expressed in terms of value per g of dry liver weight. The values are arithmetic average of 3 to 8 animals.
Keys: a, dosage (mg/animal/day); b, series I; c, series II; d, series III; e, total lipid; f, cholesterol

TABLE 5. THE EFFECT OF MYOINOSITOL ON THE SERUM TOTAL CHOLESTEROL LEVEL

a ミオイノシトール投与量 (mg/頭/日)	b シリーズI	c シリーズII	d シリーズIII
0	1.48	1.49	1.41
0.5	1.48	1.55	1.48
5	1.38	1.47	1.48
10	1.39	1.38	1.45
50	1.50	1.55	1.49

Arithmetic average of 3 - 8 animals (mg/ml)
Keys: a, dosage (mg/day/animal); b, series I; c, series II; d, series III

TABLE 6. THE EFFECT OF MYOINOSITOL ON THE LIVER LIPID P/N RATIO

ミオイノシトール投与量 (mg/頭/日) a	b シリーズI			c シリーズII			d シリーズIII		
	P (mg)	N (mg)	P/N	P (mg)	N (mg)	P/N	P (mg)	N (mg)	P/N
0	18.8	16.6	0.508±0.125	22.4	15.4	0.648±0.099	20.0	18.8	0.477±0.060
0.5	17.3	14.8	0.547±0.176	23.4	15.8	0.656±0.085	22.3	20.0	0.490±0.067
5	22.0	14.9	0.617±0.118	22.8	15.4	0.665±0.101	22.5	19.7	0.508±0.132
10	19.6	13.9	0.666±0.184	28.7	17.8	0.770±0.176	26.5	21.3	0.543±0.014
50	20.0	11.0	0.617±0.079	26.4	15.2	0.761±0.139	26.6	20.7	0.564±0.033

P and N are expressed in terms of the value per g of total lipid. The P/N indicates the molecular ratio. The values in the table indicate the arithmetic average of 3 - 8, with ± indicating the standard deviation.

TABLE 6-Keys: a, dosage (mg/animal/day); b, series I; c, series II; d, series III.

DISCUSSION

Myoinositol deficiency reportedly causes alopecia and growth retardation in mice (2), but this is not always true in higher animals in which the deficiency is rarely manifest in most instances. In fact, inositol can be synthetized in vivo to some extent (6-9) and, under no circumstance, it is expected to act as a coenzyme as other vitamin B's. Therefore, many investigators are doubtful as to the validity of treating this substance as a vitamin (25). However, we must admit the widespread recognition of the fact that it stimulates the growth of animals.

As is well known, myoinositol is found in vivo in the form of phosphatidyl inositol, among other forms. Despite its minute quantity as compared to other phospholipids, its action has drawn considerable attention in recent years.

In the light of this background, the fact that the effect of the large dose administration of myoinositol manifested as the inhibition of growth in young rats may seem to provide a key to the clarification of the nutritional significance of this compound.

Both myoinositol and choline possess affinity toward lipids, but many experiments have demonstrated that they act synergistically as well as antagonistically. The combined administration of choline and inositol to white rats reportedly exhibited greater inhibitory effect against the occurrence of fatty liver, as compared to the effect of the single administration (26). Handler (27) studied the nephric necrosis in young white rats raised on choline-deficient diet,,and found that the addition of inositol to that diet aggravated the necrosis but reduced the degree of fatty liver. Jukes (28) and Agranoff (29) conducted similar experiments with turkeys and chicks, respectively, in their stage of active growth and found that the addition of inositol to the diet increased the incidence of perosis or aggravated such condition.

In his experiment (29), Agranoff et al. observed that a large dosage of myoinositol inhibited the growth of chicks and the results of the present investigation also indicate the similar effect of the compound on young white rats. With regard to the observation, Agranoff et al. made the following analysis. During the stage of active growth, i.e., the period of active cell multiplication, the biosynthetic route of phospholipid which is indispensable to cell formation is also activated, and myoinositol and choline act antagonistically during this period. The administration of one of them at a large dosage thus causes growth retardation. The lipid biosynthetic route is shown in Table 7 for reference. According to the chart, both inositol and choline inhibit the formation of triglyceride, but they are likely to act antagonistically toward the formation of the corresponding phospholipids.

The results of the present investigation seem to support Agranoff's assumption to some degree. More specifically, the P level in the total lipid extracted from the liver reflects the total phospholipid level, and the N level, the total nitrogen-containing phospholipid level. Therefore, the increase in lipid P, consequential to the increase in the dosage of inositol, signifies an increase in the total phospholipid

level in the liver, and the drop in N, the depression of the total nitrogen-containing phospholipid level. This fact also suggests a possibility that the consecutive administration of myoinositol at a large dose during the growing stage of animals exerts some effect on the liver lipid pattern.

At this point, it is deemed necessary to call attention to the fact that the consecutive large-dose administration of myoinositol to white rats which had reached maturation exhibited no effect on their growth. This observation is indicative of the possibility that the growth inhibitive action occurs specifically during the period of active cell multiplication.

However, whether or not the fluctuation in P and N levels in the total liver lipids is directly related to the growth inhibition in white rats is yet to be determined. From the nutriological standpoint, it is necessary to investigate the effect of choline itself and whether the increased amount of N in the feed is able to compensate the inhibitive effect. What is even more important is to accurately evaluate the changes in individual liver phospholipids in each group of animals. These problems are yet to be solved, but an experiment is currently under way at this laboratory in order to clarify these problems.

CONCLUSION

The effects of myoinositol on the weight gain and liver lipid pattern in white rats were investigated.

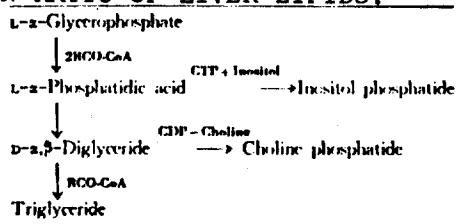
1) The large dose administration of myoinositol seems to inhibit the growth of young animals. The young animals raised on a feed containing a large dose of myoinositol showed smaller weight gain than those given feeds containing the standard dosage or substandard dosage of yoinositol. The administration of the compound at an even larger dose to mature rats produced no growth inhibitive action.

2) The examination of the liver lipid patterns of different groups of animals revealed an increased P/N ratio in the liver lipid with higher dose of myoinositol. However, no significant change was noted in the total lipid level or cholesterol level in the liver. These results suggest that the large dose administration of myoinositol influences the liver phospholipid metabolism in young white rats to some degree.

3) The mechanism of growth retardation due to a large dose of myoinositol in young animals was discussed in relation to the changes in liver lipid pattern.

The author is grateful to Prof. Yagi and Dr. Kotani of the 1st Department of Biochemistry, Nagoya University School of Medicine, for their guidance. Acknolwedgement is also due to Miss Yoshiko Yamamoto for her cooperation.

TABLE 7. THE ROUTE FOR LIPID BIOSYNTHESIS FOR THE EXPLANATION OF THE EFFECT OF A LARGE DOSE OF MYOINOSITOL ON THE P/N RATIO OF LIVER LIPIDS.



REFERENCES

1) Woolley, D. W. : J. Biol. Chem. **136**, 113(1940)
2) Woolley, D. W. : J. Biol. Chem. **193**, 29(1941)
3) 八木国夫・小滝 祥・山本良子・山路祥子・三輪ふさ子：ビタミン **30**, 17(1964)
4) Martin, G. J. : Am. J. Physiol. **136**, 124(1942)
5) Schweigert, B. S. : Vitamins and Hormones **6**, p. 55(1948)
6) Dunglady, W. H., Larner, J., Hartner, C. : J. Biol. Chem. **212**, 869(1955)
7) Harriday, J. W., Anderson, L. : J. Biol. Chem. **217**, 797(1955)
8) Imai, Y. : J. Biochem. **53**, 50(1963)
9) Eisenberg, F., Jr., Bolden, A. H. : Biochem. Biophys. Res. Comm. **12**, 72(1963)
10) Eagle, H., Oyama, V.I., Levy, M., Freeman, A. E. : J. Biol. Chem. **226**, 191(1957)
11) Charalampous, F., Wahl, M., Ferguson, L. : J. Biol. Chem. **236**, 2552(1961)
12) Webb, S. J., Cormack, D. W., Morrison, H. G. : Nature **201**, 1103(1964)
13) Webb, S. J., Cook, C. A. M., Bather, R. : Canadian J. Biochem. **42**, 157(1964)
14) Bather, R., Sebastian, I., Webb, S. J. : *ibid.* **42**, 167(1964)
15) Vignais, P. M., Vignais, P. V., Lehninger, A. L. : J. Biol. Chem. **239**, 2011(1964)
16) Hawthorne, J. N., Kemp, P. : Adv. Lipid Res. **2**, p. 127(1964)
17) Forker, B. R., Morgan, A. F. : J. Biol. Chem. **209**, 303(1954)
18) Entenman, C. : Methods in Enzymology **3**, p. 299(1957)
19) Allen, R. J. L. : Biochem. J. **34**, 858(1940)
20) 中村道徳：農芸化学会誌 **24**, 1(1950)
21) 八木国夫・奥田 潤：蛋白質核酸酵素 **4**, 57 (1959)
22) Zlatkis, A., Zak, B., Boyle, A. J. : J. Lab. Clin. Med. **41**, 486(1953)
23) 丹羽壮一・小滝 祥・八木国夫：ビタミン **30**, 23(1964)
24) Scott, T. A., Jr., Melvin, E. H. : Anal. Chem. **25**, 1656(1953)
25) Novelli, G. D. : Ann. Rev. Biochem. **26**, 243 (1957)
26) Forbes, J. C. : Proc. Soc. Exp. Biol. Med. **54**, 89(1943)
27) Handler, P. : J. Nutr. **31**, 621(1946)
28) Jukes, T. M. : Poulty Sci. **20**, 251(1941)
29) Agranoff, B. W., Fox, M. R. S. : Nature **183**, 1259(1959)

3. Yagi, K., S. Kotaki, R. Yamamoto, K. Yamaji, and F. Miwa: Bitamin, Vol. 30, p. 17, 1964.
20. Nakamura, M.: Nogei Kagaku Kaishi, Vol. 24, p. 1, 1950.
21. Yagi, K., and J. Okuda: Tanpakushitsu Kakusan Koso, Vol. 4, p. 57, 1959.
23. Niwa, S., S. Kotani, and K. Yagi: Bitamin, Vol. 30, p. 23, 1964.

Biochem. J. 37: 709-712, 1943

The Estimation of Inositol in Animal Tissues

By B. S. PLATT AND GERTRUDE E. GLOCK, *Nutrition Building, National Institute for Medical Research, Mill Hill, N.W. 7, and the Lister Institute of Preventive Medicine, Chelsea Bridge Road, S.W. 1*

(*Received* 31 *July* 1943)

Inositol occurs in animal tissues in both free and combined forms. The occurrence of water-soluble combined inositol was first suggested by Rosenberger [1908*a*,*b*, 1910]. Comparing the inositol contents of fresh rabbit muscle and commercial beef muscle, he came to the conclusion that animal tissues contain 'inositogen'. This may be identical with the 'tyrophenosit' of Danileffski [1884], who reported, without giving data, a considerable increase in the inositol content of mice after 6 days' autolysis at 37° in the presence of $CHCl_3$. Needham [1923] found that the skeletal muscle of a freshly killed rabbit contained only one-half the amount of inositol found in that of a rabbit killed 2 days previous to the estimation. An increase in the free inositol content of the skeletal muscle of rats occurred after autolysis of the tissues [Needham, 1924]. Winter [1934], working on cardiac muscle of dogs, postulated the occurrence of three forms of inositol in animal tissues: 'free' inositol, determined after ½ hr. boiling with 10% KOH; 'combined' inositol, determined after 5 hr. boiling with 10% KOH, and 'newly formed' inositol, liberated after incubation at 37° in nitrogen. The cardiac muscle of the sheep, ox and pig contained no 'combined' inositol [Winter, 1940]. It is doubtful, however, whether much significance can be attached to any of Winter's results, owing to the unreliability of the chemical methods he employed. Woolley [1941*a*] obtained inositol after acid or alkaline hydrolysis of an ethanol-insoluble, H_2O-soluble, non-dialyzable substance isolated from liver. This was considered to be probably a phosphoric acid ester of inositol. Rapoport [1940] found that in bird and turtle erythrocytes a large proportion of the organic acid-soluble P is present in the form of phytic acid. Besides being present in animal tissues in water-soluble combined forms, inositol also occurs in brain and spinal cord bound in a phosphatide fraction. Folch & Woolley [1942] isolated inositol from an acid hydrolysate of brain cephalin, and found that it constituted up to 10% of this phosphatide fraction and amounted to as much as 0·4% of the weight of the brain.

It is evident that further work is required to elucidate the nature of the water-soluble combined forms of inositol in the animal body and the methods of breakdown and significance of both water-soluble and water-insoluble combined forms. Recent developments have, in fact, followed on the application of a microbiological technique [Williams, Stout, Mitchell & McMahan, 1941; Woolley, 1942]. The chemical methods at present available for the estimation of inositol in biological material [see Needham, 1923, 1926; Winter, 1934, 1940; Young, 1934*a*,*b*; and Gregory, 1935] are, however, tedious, lengthy and involve the use of relatively large amounts of tissues. The method to be described is much quicker than previous ones and possesses several other advantages, including improvements in the extraction of inositol from tissues, the removal of interfering substances by ion exchange materials and the estimation of inositol, without isolation, by oxidation with periodic acid.

METHODS

Principle of the method of estimation

Dried tissue is extracted with water and the fractions insoluble in 70% acetone and soluble in ether both removed from the aqueous extract. Glucose is then removed by yeast fermentation and both acidic and basic substances present in the extract removed by adsorption on a mixture of ion exchange adsorbents. The free inositol present in the extract is then quantitatively oxidized with HIO_4, the excess HIO_4 being estimated iodometrically. Suitable corrections are applied for any HIO_4 used in oxidizing the glycerol which may be present in the extract at this stage.

Water-soluble combined inositol is determined after acid hydrolysis of the aqueous extract.

Experimental

Preparation of tissue for extraction. As soon as possible after death, the tissue is removed from the animal, sectioned on a freezing microtome and the sections dried in thin layers in Petri dishes *in vacuo* in the cold room over silica gel. Drying is completed in an oven at 110°, after which the material is powdered and kept in the cold until analyzed. The water content of another portion of the same tissue is determined by drying to constant weight at 110°.

Extraction of inositol. An amount of dried powdered tissue containing approximately 0·5–1·0 mg. inositol (i.e. 1·0–2·0 g. dry skeletal muscle powder) is brought to the boil with 50 ml. distilled water, filtered under reduced pressure through a small plug of cotton-wool in a funnel, and the vessel and filter washed with two portions of 5 ml. of distilled water. This process is repeated twice, the three

extracts pooled and evaporated to about 50 ml., and acetone added to give a final concentration of 70%. The precipitate is filtered off and the acetone removed from the filtrate by distillation. The extract is evaporated down to about 25 ml. and extracted with ether. The ether extract is washed once with about 20 ml. distilled water, and the washings added to the aqueous residue, which is then concentrated to approximately 30 ml. If it is desired to estimate the 'combined' water-soluble inositol as well as the 'free' inositol the aqueous extract can be divided into two portions and one hydrolyzed as described below.

Removal of glucose. The only satisfactory method for the removal of glucose was found to be yeast fermentation, as used by Young [1934*a*]. Since some products of glucose fermentation have to be removed on the ion exchange adsorbents, the fermentation stage was carried out before the adsorption.

A washed yeast suspension is prepared by shaking one part by weight of pressed baker's yeast with five parts of distilled water, and centrifuging; this process is repeated four times and the suspension of washed yeast then made up to the original volume with distilled water. The suspension is prepared fresh each day and its potency tested by incubating 2 ml. with 25 ml. glucose solution (containing 5 mg. glucose) for 10 min. at 37°, and estimating the reducing power of the supernatant fluid, obtained by centrifuging, by Hagedorn & Jensen's ferricyanide method, a blank being set up at the same time with yeast suspension and water only.

The tissue extract is incubated at 37° with 2 ml. of the yeast suspension for 10 min. and then centrifuged. The supernatant liquid is siphoned into a boiling tube and the yeast washed twice by centrifuging with 10 ml. portions of distilled water, the washing being kept separate from the main supernatant fluid for the subsequent adsorption.

Preparation of ion exchange materials and the removal of interfering substances from tissue extracts. After treatment with yeast, the extract is purified by the use of ion exchange materials, with a carbonaceous zeolite for cation exchanges and 'M.P.D. Resin' as an acid adsorbent. Both these are obtainable from Permutit Company, Limited. A brief account of the use of these substances for the purification of muscle extracts has been published [Platt & Glock, 1942]. The removal of creatine and creatinine from muscle extracts by this method proved to be much more efficient than by the precipitation technique of West & Petersen [1932] with $HgSO_4$ and $BaCO_3$, which was employed by Young [1934*b*]. Treatment of the extract with a mixture of regenerated, washed and dried carbonaceous zeolite and 'M.P.D. Resin' is more efficient than successive treatments with the two materials separately. With this technique, however, the adsorbing materials cannot be regenerated.

250 g. or more of the carbonaceous zeolite are shaken gently at intervals for a period of 1 hr. with 2 vols. of 2N HCl, filtered on a Buchner funnel and washed on the funnel with a total volume of approximately 5 l. of distilled water. The final washings should be neutral to litmus. This regenerated, washed cation exchange material is dried at 100° and sieved so that the particles are of 20/40 mesh sieve size. The 'M.P.D. Resin' is treated in a similar way, but with 2N NH_4OH instead of HCl. At least 1 l. of hot distilled water should be used to wash this reagent, in order to remove excess *m*-phenylene diamine which is liable to be present and which, if not removed completely, gives a blank reading with HIO_4. These two regenerated, washed, dried and sieved materials are mixed in equal parts by weight; this preparation will be called the 'adsorbent'.

15 g. of adsorbent are introduced into a tube approximately 20 in. by $\frac{7}{16}$ in., narrowed at the bottom to approximately $\frac{5}{16}$ in., and fitted with a small piece of rubber tubing carrying a glass tip and provided with a screw clip. The material is kept in position in the tube with a loosely packed plug of glass-wool. Before being treated with the tissue extract, the adsorbent is washed with a total volume of approximately 100 ml. of distilled water. The final washings must give no blank when allowed to react with 5 ml. 0·01 M HIO_4 for 10 min. at room temperature. To prevent the formation of air bubbles in the column, which would impede the passage of liquid, the tube should contain distilled water into which the adsorbent is poured, the level of the liquid being kept above the top of the adsorbent. After the adsorbent has been washed sufficiently, the supernatant liquid from the yeast treatment is poured into the tube and allowed to drip through the column at a rate such that the whole of the liquid passes through in approximately 90 min. When the liquid has passed through completely, the first 10 ml. portion of the yeast washings is poured into the tube, and the rate of flow adjusted so that this liquid passes through in approximately 15 min. The tube containing the column of adsorbent is sucked dry on a filter pump, and then a second 10 ml. portion of yeast washings is allowed to drip through the column, which is then sucked dry. The column is washed with the help of suction with 15 successive 5 ml. portions of distilled water. This amount of washing is found to be necessary and sufficient to obtain quantitative recovery of 1 mg. of added inositol.

The assumption has been made that the only HIO_4-reacting substances which are not removed from yeast-fermented muscle extracts by the adsorbent mixture are inositol and glycerol.

Oxidation with HIO_4. At the suggestion of Prof. H. Raistrick, we investigated the possibility of estimating inositol by oxidation with HIO_4. Malaprade [1928*a*, *b*, 1934] found that polyhydroxy alcohols were oxidized quantitatively by HIO_4 at room temperature. Fleury & Joly [1937*a*, *b*] used periodate for the estimation of inositol both in pure solution and in the presence of glucose, but under the conditions used by them the oxidation, when allowed to go to completion, used up much more periodate than was to be expected from the equation

$$C_6H_6(OH)_6 + 6HIO_4 = 6HIO_3 + 6H.COOH,$$

and they came to the conclusion that secondary reactions occurred.

By allowing the reaction to go to completion at low temperatures (6–8°), we have found that the utilization of HIO_4 is quantitative. Formic acid, determined by titrating to pH 5·5 with 0·01 N NaOH, with a mixture of chlorophenol red and bromocresol green as indicator, has been produced to the extent of about 90% of the theoretical amount by the time the utilization of HIO_4 has reached the theoretical figure. Thereafter no more formic acid is formed. Some typical results for the reaction between HIO_4 and inositol in varying concentrations at 8 and 30° are given in Fig. 1.

Differential oxidation of inositol and of glycerol by HIO_4. After the removal of glucose from muscle extracts by fermentation with yeast, small amounts of glycerol remain. Some, no doubt, is extracted from the muscle and an

amount equivalent to approximately 4% of the glucose fermented is produced by fermentation. Attempts to remove glycerol by making use of its slight solubility in ether, its reported solubility in dry ethyl acetate, and its fermentation by *Bacillus proteus*, all failed. Glycerol, in concentrations of 0·2–0·9 mg./100 ml. solution, is, however, oxidized completely and quantitatively by 2 ml. 0·01 M HIO_4 at 8° in 90 min.

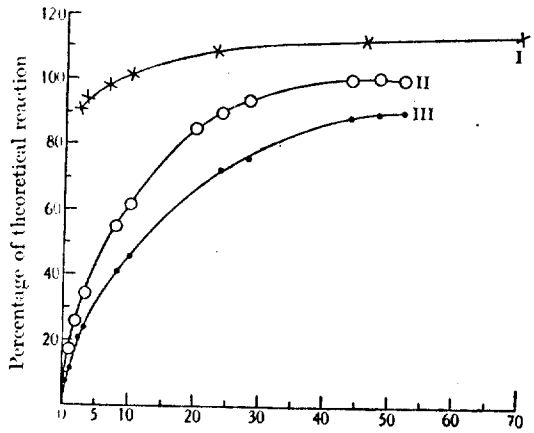


Fig. 1. Reaction of inositol with periodic acid. Extent to which the reaction

$$C_6H_6(OH)_6 + 6HIO_4 = 6H.COOH + 6HIO_3$$

is followed.

I, 1 mg. inositol/10 ml. + 5 ml. 0·01 M HIO_4 at 30°.
II, 1 mg. inositol/100 ml. + 10 ml. 0·01 M HIO_4 at 8°.
III, 1 mg. inositol/100 ml. + 5 ml. 0·01 M HIO_4 at 8°.

The percentage oxidation of glycerol (0·8 mg./100 ml.) by 2 ml. 0·01 M HIO_4 at 8° at intervals up to 100 min. is as follows:

Time in min.	20	30	40	60	70	80	90	100
% oxidation	53·0	68·6	79·8	95·6	98·0	100	100	100

The time for complete oxidation of glycerol depends on the temperature at which the reaction is carried out and on the concentration of both glycerol and HIO_4: e.g. at 6° 1 mg. of glycerol in 10 ml. water is completely oxidized by 3·0 ml. 0·01 M HIO_4 in 15 min.

Concentrations of inositol of 0·5–2·5 mg./100 ml. react to the extent of 1·7–2·2% with 2·0 ml. 0·01 M HIO_4 at 8° in 90 min. It was therefore necessary to determine (*a*) the total HIO_4-reacting material (inositol plus glycerol) in 100 ml. of solution treated with 10 ml. 0·01 M HIO_4 at 8° for 48 hr., and (*b*) the material reacting under the same conditions of concentration and temperature with 2 ml. 0·01 M HIO_4 in 90 min. (*b*) includes the whole of the glycerol and approximately 2% of the total inositol. Thus the difference between (*a*) and (*b*) multiplied by 1·02 gives the inositol content of the solution.

Absence of HIO_4-reducing substances other than glycerol and inositol from fermented effluent. There is support for the assumption, that glycerol and inositol are the only HIO_4-reacting substances left after fermentation and adsorption, in the fact that the glycerol content of the purified extracts, calculated from the HIO_4 reduced under the conditions stated above, is identical with that calculated from the formaldehyde formed, estimated as the dimedone derivative. The only other possible substances which would react with HIO_4 are amino-acids. Tests for NH_3 production (from β-hydroxy amino-acids) with HIO_4 in strongly alkaline solution in a Conway unit were always negative, as also were tests for amino-N by formol titration.

Estimation of free inositol. The effluent from the adsorption procedure is made up to 200 ml. in a graduated flask and 100 ml. withdrawn. Both portions are cooled to 8°. To one portion are added 10 ml. 0·01 M HIO_4 (also at 8°) and the reaction allowed to proceed for 48 hr. To the other portion, 2 ml. HIO_4 are added and the reaction is allowed to proceed at 8° for 90 min. If a temperature other than 8° is selected the time relationships for oxidation of both glycerol and inositol at this temperature must be determined. The reaction is stopped in both cases by adjusting the pH to approximately 6·5 by the addition of 5 ml. phosphate buffer (12 g. $Na_2HPO_4.12H_2O$ and 20 ml. N H_2SO_4/100 ml.), followed by 5 ml. 5% KI solution. After 5 min. the I_2 liberated is titrated with approximately 0·004 N $Na_2S_2O_3$ with 1% soluble starch in saturated NaCl as indicator, the end-point being stabilized by the addition of a few drops of saturated $NaHCO_3$ solution. 1 mg. inositol contained in 100 ml. distilled water is also allowed to react with 10 ml. 0·01 M HIO_4 for 48 hr. at 8°, and the excess HIO_4 titrated as above.

The HIO_4 reduced is obtained from the difference between the titration of the purified tissue extract after reaction with HIO_4 and the blank titration obtained with the HIO_4 alone.

Let x = HIO_4 reduced in terms of 0·004 N $Na_2S_2O_3$ in 48 hr. at 8°,

y = HIO_4 reduced in terms of 0·004 N $Na_2S_2O_3$ in 90 min. at 8°,

z = HIO_4 reduced by 1 mg. inositol in 48 hr. at 8°.

The approximate inositol content in mg. of half the extract is given by $(x-y)/z$ which should be multiplied by 1·02 (see above).

Estimation of 'combined inositol'. For the estimation of the water-soluble combined inositol, the extract is hydrolyzed with acid [Woolley, 1941*b*]. Both water-soluble fractions (i.e. 'free' and 'combined') are determined in the same sample of tissue, with twice as much tissue as in the procedure outlined above. Before fermentation, the extract is made to 50 ml., and one-half is refluxed for 6 hr. with HCl (final concn. 18% HCl). The HCl is removed by distillation under reduced pressure, and the contents of the flask are washed out with distilled water to give a total volume of approximately 25 ml. and then neutralized by the dropwise addition of N NaOH.

Experimental diets

Albino rats were put on an experimental diet, containing only small amounts of inositol, at 6 weeks of age and were in good condition when killed after an experimental period of 16½ months. The diet consisted of casein (A.E. Glaxo), 24%; sucrose, 67·5% (replaced by maize dextrin, after 13½ months on diet, for 2 months and by rice starch for last month); McCollum's salt mixture (185), 4%; wheat-germ oil, 2%; dried yeast (*Torula utilis*, 95% dry matter, containing not more than 2·7 mg. inositol/g.), 2·5%, in-

creased to 5% after 2 months on the diet; ascorbic acid 2·5 mg. daily; vitamin A approximately 22·5 I.U. daily and vitamin D approximately 4·5 I.U. daily (both contained in 2 drops of Radiostoleum diluted with peanut oil). Between 8 and 12·5 g. of this diet, mixed to a thick paste with water, were fed daily to each rat.

RESULTS

Inositol content of rat tissues

Table 1 contains the mean results of analyses of the tissues of four rats for water-soluble inositol, free and total (i.e. including combined).

Table 1. *Inositol content of rat tissues*

	Water-soluble inositol (mg./100 g. tissue)			
	Free		Total	
Tissue	Dry wt.	Wet wt.	Dry wt.	Wet wt.
(*a*) Rats given 5 mg. inositol/day				
Skeletal muscle	49·3	12·9	81·2	21·3
Liver	85·2	24·8	178	52·1
Heart	141·3	34·3	186	45·3
Brain	241	55·8	286	67·0
Kidney	328	88·0	456	123
(*b*) Rats given no extra inositol				
Skeletal muscle	24·0	6·3	76·2	18·3
Liver	89·0	25·5	174	50·3
Heart	—	—	—	—
Brain	169	37·8	295	66·1
Kidney	316	79·5	426	113

Our values for the total water-soluble inositol contents of rat tissues are of the same order as those found by other workers, including those recently obtained by Williams *et al.* [1941], with a microbiological method, on tissue autolysates. The proportions of free to total water-soluble inositol are also of the same order as those obtained by Woolley [1942], who also used a microbiological method.

It will be seen that there is no important difference between the values for the total water-soluble inositol for the rats receiving the inositol-deficient diet, and those with added inositol. It thus appears that rats are able to synthesize inositol, which confirms the earlier work of Needham [1924]. Woolley [1942] also showed that mice could synthesize inositol in the gut if the diet was not deficient in pantothenic acid.

SUMMARY

1. A chemical method is described for the estimation of inositol in animal tissues. This possesses advantages over earlier methods in that the time taken for an estimation is considerably reduced, the method of extraction of inositol from the tissues improved, the tedious precipitation procedures replaced by treatment with ion exchange materials, and the inositol finally estimated in solution by reaction with HIO_4 without isolation of the inositol.

2. Both free and total water-soluble inositol were determined in the kidney, heart, liver, brain and skeletal muscle of control rats, and of rats receiving a daily supplement of 5 mg. inositol. No appreciable difference was found between the total water-soluble inositol contents of the tissues in the two groups of rats.

REFERENCES

Danileffski, B. [1884]. *Bull. Soc. chim. Paris*, **41**, 255.
Fleury, P. & Joly, M. [1937*a*]. *J. pharm. chim.* **26**, 341.
—— —— [1937*b*]. *J. pharm. chim.* **26**, 397.
Folch, J. & Woolley, D. W. [1942]. *J. biol. Chem.* **142**, 963.
Gregory, R. A. [1935]. *Biochem. J.* **29**, 2798.
Malaprade, L. [1928*a*]. *C.R. Acad. Sci., Paris*, **186**, 238.
—— [1928*b*]. *Bull. Soc. chim. Fr.* (4th ser.), **43**, 683.
—— [1934]. *Bull. Soc. chim. Fr.* (5th ser.), **1**, 833.
Needham, J. [1923]. *Biochem. J.* **17**, 422.
—— [1924]. *Biochem. J.* **18**, 891.
—— [1926]. *Ergebn. Physiol.* **25**, 1.
Platt, B. S. & Glock, G. E. [1942]. *Biochem. J.* **36**, *Proc.* xviii.
Rapoport, S. [1940]. *J. biol. Chem.* **135**, 403.
Rosenberger, F. [1908*a*]. *Hoppe-Seyl. Z.* **56**, 373.
—— [1908*b*]. *Hoppe-Seyl. Z.* **57**, 464.
—— [1910]. *Hoppe-Seyl. Z.* **64**, 341.
West, E. S. & Peterson, V. L. [1932]. *Biochem. J.* **26**, 1720.
Williams, R. J., Stout, A. K., Mitchell, H. K. & McMahan, J. R. [1941]. *Studies on the Vitamin Content of Tissues*, **1**. University of Texas Publication No. 4137. University of Texas, Austin, Texas.
Winter, L. B. [1934]. *Biochem. J.* **28**, 6.
—— [1940]. *Biochem. J.* **34**, 249.
Woolley, D. W. [1941*a*]. *J. biol. Chem.* **139**, 29.
—— [1941*b*]. *J. biol. Chem.* **140**, 453.
—— [1942]. *J. exp. Med.* **75**, 277.
Young, L. [1934*a*]. *Biochem. J.* **28**, 1428.
—— [1934*b*]. *Biochem. J.* **28**, 1435.

Biochem. Jour. 40:(4) 494–499, 1946

491

Lipotropic Factors and the Fatty Liver Produced by Feeding Cholesterol

By JESSIE H. RIDOUT, COLIN C. LUCAS, JEAN M. PATTERSON and C. H. BEST
Banting and Best Department of Medical Research, University of Toronto

(*Received* 1 *May* 1946)

The excessive deposition of liver lipids which is produced in rats by feeding diets containing cholesterol is inhibited by choline and betaine (Best & Ridout, 1933; Best, Channon & Ridout, 1934). Choline was shown to affect both glycerides and cholesteryl esters but deposition of the former was inhibited more than that of the latter. Many preventive and curative experiments were subsequently reported and the results were in general agreement with those of the earlier investigations.

In the early studies no evidence of any significant waning in the lipotropic effect of choline was observed with or without cholesterol in the diet (Best & Ridout, 1936). Himsworth & Glynn (1944), however, report that this occurs in cholesterol-fed rabbits under certain experimental conditions. Furthermore, it has been stated by McHenry & Patterson (1944) that inositol but not choline has a definite effect on cholesterol metabolism. It became evident, therefore, that further studies were necessary to determine (*a*) the relative lipotropic effects of choline and inositol, and (*b*) the duration of the action of these agents when fed separately and in combination.

These problems have been reinvestigated in cholesterol-fed rats observed for periods up to 16 weeks. The effectiveness of choline in preventing deposition of cholesteryl esters did not diminish during this period, while that of inositol, which initially was definitely less than that of choline, progressively decreased. Under certain conditions inositol exerted no lipotropic action.

EXPERIMENTAL

White rats of the Wistar strain, reared in our own colony, were used. A few days before an experiment was started the animals were placed in individual cages, with a false bottom of coarse wire screen, in order to accustom them to the environment. The groups in each experiment were then balanced as far as possible with respect to weight and sex.

Weighed amounts of fresh diet were given daily, in feed trays designed to minimize spilling, and the following morning the amount left over and the scatter were weighed. From these data the individual daily food consumptions were calculated. The average values are recorded in the Tables. The rats were 'group pair-fed' and cared for as described previously (Best, Lucas, Patterson & Ridout, 1946).

The composition of the basal diets is given in Table 1. The supplements used in the test diets are shown in Tables 2–4. Choline was incorporated as its chloride, in molecularly equivalent amount, e.g. 0·345% choline chloride was used in diets stated to contain 0·30% choline.

In Exp. 1 the test diets (see Table 2) were fed for periods of 3, 8 and 16 weeks in order to determine whether any

Table 1. *Percentage composition of basal diets*

Component	Content (%) in diet nos. 1	1A	2	2A	3	3A	3B	3C	3D
Casein	8	8	8	8	10	10	10	10	10
Gelatin	12	12	12	12	—	—	—	—	—
Sucrose	60	59·5	73	70	84	81	51	51	51
Salts	5	5	5	5	4	4	4	4	4
Cellu flour	2	2	2	2	2	2	2	2	2
Beef dripping	10	10	0	0	0	0	30	—	20
Corn oil	2	2	0	2	0	0	—	30	10
Vitamin powder*	1†	1†	‡	‡	0	1	1	1	1
C.L.O. concentrate§	0·010	0·010	0·010	0·010	0·015	0·015	0·015	0·015	0·015
Cholesterol	0	0·5	0	1	0	2	2	2	2

* The vitamin powder consisted of aneurin hydrochloride 500 mg., riboflavin 250 mg., pyridoxin 200 mg., calcium pantothenate 1000 mg. and nicotinic acid 1000 mg. made to 1000 g. with very finely powdered (100 mesh) sucrose. Rat eating 10 g. of food per day receive 50 μg. aneurin hydrochloride and proportional amounts of the other vitamins daily.

† Diets 1 and 1A contained not only the usual B vitamins but also per 10 g. diet, 1·5 mg. vitamin E (*x*-tocopheryl acetate) and 50 μg. vitamin K (2-methyl 1, 4-naphthoquinone).

‡ The rats received daily subcutaneous injections of the following B vitamins in 0·5 ml. physiological saline; aneurin hydrochloride 50 μg., riboflavin 25 μg., pyridoxin 20 μg., calcium pantothenate 100 μg. and nicotinic acid 100 μg.

§ The cod-liver oil concentrate (Ayerst, McKenna & Harrison, Ltd. Montreal), contained 200,000 i.u. vitamin A and 50,000 i.u. vitamin D/g.

waning of the lipotropic effect of choline or inositol occurred during this period. In Exp. 2, of duration 8 weeks, a comparison was made of the relative effectiveness of choline and inositol, singly and in combination, when fed at several different levels, in diets with and without added cholesterol (Table 3). The rats in Exp. 3 were fed a ration devoid of B vitamins for a preliminary period of 3 weeks. Comparable groups of these animals were then fed the cholesterol-containing test diets (Table 4) for 1 week to examine the effect of the preliminary period of vitamin depletion (similar to that used by McHenry and his associates) upon the relative efficacy of the lipotropic factors.

The animals were stunned and the livers removed immediately. The liver lipids were extracted with hot absolute ethanol and analyzed as described by Best *et al.* 1946.

RESULTS

The data for total lipids (Tables 2–4) are presented as percentage of wet liver weight for ease of comparison with many values in the literature. The individual components of the liver lipids are reported as mg./liver. From these values and the other data in the Tables, each component may be calculated to either a wet weight or fat-free dry residue weight basis. For many purposes the latter method of expression is to be preferred. The magnitude of normal values for liver lipids expressed as percentage of dry, fat-free residue weight, was determined to serve as a base for comparison. Livers of ten rats (125–160 g.) on a stock diet (Master fox breeder mixture), analyzed individually, gave: total lipids average 25·3 (range 20·4–31·6); phospholipids 11·1 (9·2–12·9); free cholesterol 0·87 (0·73–0·98); total cholesterol 1·02 (0·90–1·21); cholesteryl esters, calculated as oleate 0·26 (0·18–0·36); glyceride 12·4 (7·8–17·7). A summary of the more pertinent data, calculated on the basis of fat-free dry residue, is given in Table 5.

DISCUSSION

Exp. 1. The results (Tables 2 and 5) provide further evidence that the lipotropic action of choline is not evanescent. They do not support the claim that inositol is more effective than choline upon cholesteryl esters.

Total liver lipids of rats on the cholesterol-free ration (diet 1, groups 1, 5 and 9) continued to increase beyond the three-week period frequently used in previous studies. The value obtained at 8 weeks was, however, just as high as at 16 weeks. As the glycerides accumulated in the liver, cholesteryl esters tended to rise proportionately, whether dietary cholesterol was present or not (cf. also groups 2, 6 and 10, in which cholesterol was present in the diet). Comparison of these two sets of data shows that the amount of cholesterol in the diet affects the ratio in which cholesteryl esters and glycerides are deposited. A graphic representation

Table 2. *Effects of dietary supplements on the rate of deposition and composition of liver lipids*

(Average initial weight of rats 150 g. (range 125–175 g.).)

Basal diet no.	Group no.	Addition to diet: Substance	Addition to diet: %	Survivors/Starters	Average food intake (g./day)	Liver: Weight change (%)	Liver: Average wet weight (g.)	Liver: Average fat-free dry residue weight (g.)	Liver: Total lipids (% wet liver wt.)	Composition of liver lipids (mg./liver): Total lipids	Phospholipids	Cholesterol: Free	Cholesterol: Total	Cholesterol: Ester*	Glyceride
Test diets fed for 3 weeks															
1	1	None	—	12/12	10·4	+ 2	10·4	1·748	27·19	2833	210	14·6	31·7	29	2579
1A	2	None	—	11/12	10·6	5	11·3	1·720	37·69	4248	248	20·9	147·7	214	3765
	3	Choline	0·3	10/12	11·5	12	8·5	1·779	8·38	712	178	16·5	89·6	123	394
	4	Inositol	0·3	11/12	10·9	3	11·1	1·651	30·35	3373	248	20·0	117·5	164	2941
Test diets fed for 8 weeks															
1	5	None	—	8/12	10·1	+ 3	13·3	1·837	37·50	4973	236	20·4	78·1	97	4619
1A	6	None	—	9/12	10·6	9	16·2	2·075	37·59	6089	280	37·7	412·1	631	5141
	7	Choline	0·3	10/12	9·9	27	7·8	1·748	8·08	627	236	16·8	91·7	126	248
	8	Inositol	0·3	7/12	10·0	6	13·4	1·825	39·31	5246	270	33·6	273·9	405	4538
Test diets fed for 16 weeks															
1	9	None	—	10/12	10·0	+ 15	14·6	2·042	36·68	5420	247	23·5	78·5	93	5057
1A	10	None	—	9/12	10·0	8	16·7	2·051	38·66	6463	230	44·5	490·3	753	5436
	11	Choline	0·3	9/12	10·4	54	8·7	2·101	7·71	672	253	18·8	73·7	92	308
	12	Inositol	0·3	8/12	10·3	33	14·9	2·042	36·29	5418	246	34·4	400·7	617	4521

In this and subsequent Tables, the cholesteryl ester value has been expressed as cholesteryl oleate, obtained by multiplying the weight of bound cholesterol by the factor 1·684.

Table 3. *Lipotropic effects of choline and inositol with and without cholesterol*

Basal diet no.	Group no.	Addition to diet: Substance	Addition to diet: %	Survivors/ Starters	Average food intake (g./day)	Weight change (%)	Liver: Average wet weight (g.)	Liver: Average fat-free dry residue weight (g.)	Liver: Total lipids (% wet liver wt.)	Composition of liver lipids (mg./liver): Total lipids	Phospholipids	Cholesterol: Free	Cholesterol: Total	Cholesterol: Ester*	Glyceride
2*	13	None	—	21/22	11·5	0	10·5	1·584	31·24	3238	240	18·6	77·7	100	2879
	14	Choline	0·1	9/10	11·4	+14	7·9	1·792	8·30	659	266	13·8	26·1	21	359
	15	Choline	0·5	12/12	11·3	+ 9	7·0	1·613	8·26	582	273	15·0	24·6	16	278
	16	Inositol	0·1	9/10	10·6	+ 4	8·0	1·442	21·81	1744	228	14·8	38·9	41	1461
	17	Inositol	0·3	11/12	11·4	− 4	8·7	1·615	23·34	2084	264	17·6	41·6	40	1762
	18	Choline + inositol	0·1 + 0·1	9/10	11·5	+19	6·7	1·510	7·18	483	249	13·3	21·7	14	208
	19	Choline + inositol	0·1 + 0·3	10/10	10·9	+17	7·0	1·617	6·44	453	239	12·8	18·8	10	191
	20	Choline + inositol	0·5 + 0·3	11/12	11·7	+10	6·7	1·615	6·20	415	252	15·0	18·2	5	142
2A*	21	None	—	20/22	11·3	+ 4	12·1	1·591	39·71	4824	283	28·9	316·7	485	4027
	22	Choline	0·1	10/10	10·8	+26	7·0	1·493	15·00	1056	280	16·5	112·5	162	597
	23	Choline	0·5	11/12	11·4	+ 6	6·2	1·454	8·95	556	241	16·4	56·8	68	231
	24	Inositol	0·1	10/10	10·8	+18	11·2	1·356	34·86	3915	265	23·0	250·0	382	3245
	25	Inositol	0·3	11/12	11·1	+ 1	11·4	1·523	36·04	4109	275	26·4	231·8	346	3462
	26	Choline + inositol	0·1 + 0·1	10/10	11·2	+25	6·6	1·481	12·20	808	271	17·0	118·9	168	352
	27	Choline + inositol	0·1 + 0·3	9/10	10·5	+21	6·0	1·375	10·28	620	241	14·3	58·3	74	290
	28	Choline + inositol	0·5 + 0·3	11/12	11·9	+11	6·5	1·555	7·52	485	275	16·4	30·7	24	169

* Limited availability of animals necessitated doing the experiment in two sections with a time interval of about a month. The average weight of the 12 animals used in each group in the first part was 175 g. (range 140–220 g.) and of the 10 in the second part was 140 g. (range 80–200 g.). The difference in size of rats used in the two parts of the experiment did not affect appreciably the total liver lipids (the values in the two basal groups were: diet 2, 33·11 and 28·74, diet 2A, 38·83 and 40·78% of wet liver weight) or the composition of the lipids. The figures given in the Table for basal Groups 13 and 21 are weighted averages of the two sets of values.

of another example of this relationship has been published recently (Best *et al.* 1946).

When the basal diet contained cholesterol (groups 2, 6 and 10) the liver lipids appeared to reach a maximum within 3 weeks, if calculated as percentage of wet liver weight. If expressed as percentage of fat-free dry residue weight, however, it will be noted (Table 5) that the liver lipids continued to increase throughout the test period, the values at 3, 8 and 16 weeks being 247, 294 and 315%, respectively. This presumably means that, after 3 weeks, fat and water tend to be deposited in the same ratio. The increase in fat was due mainly to accumulation of glycerides with smaller increments due to cholesteryl esters.

The inclusion of 0·3% choline (groups 3, 7 and 11) in the diet kept the total liver lipids at a low level (about 8% of wet liver weight, which is only slightly above normal) throughout the whole 16-week period.* Inositol fed at the same level (groups 4, 8 and 12) was never nearly as effective as choline. At 3 weeks choline had reduced total lipids from 247 to 40% of fat-free dry residue weight, while inositol had brought the value down only to 204%, and at 16 weeks the influence of inositol was even less (Table 5).

Free cholesterol showed a small and possibly insignificant rise in the livers of the animals on the cholesterol-free ration (diet 1, groups 1, 5 and 9) as the experiment was prolonged (Table 5). The presence of 0·5% cholesterol (diet 1A, groups 2, 6 and 10) caused a slightly greater and probably significant increase in the free sterol. Chanutin & Ludowig (1933) have also noted increases in free cholesterol after feeding cholesterol under somewhat different conditions. Choline tended to restore the level of free cholesterol in the liver to normal (Table 5), while inositol had a negligible effect.

The cholesterol intake (0·5%), while considerably smaller than in some previous experiments, was still greatly in excess of the amount contained in the normal diet of rats. While cholesteryl esters were not reduced to normal values by 0·3% choline there was a marked and progressive reduction,

* Himsworth & Glynn (1944) noted an evanescent effect of 4 mg. choline daily in rats fed a diet containing 50% lard. Data to be published shortly indicate that this amount of choline is insufficient to maintain liver fat at low levels under these conditions.

Table 4. *Effect of preliminary depletion of B vitamins on lipotropic activity*

(Average initial weight of rats at beginning of test period 65 g. (range 55–85 g.).)

Basal diet no.	Group no.	Addition to diet: Substance	Addition to diet: %	Survivors/ Starters	Average food intake (g./day)	Weight change (%)	Liver: Average wet weight (g.)	Liver: Average fat-free dry residue weight (g.)	Liver: Total lipids (% wet liver wt.)	Composition of liver lipids (mg./liver): Total lipids	Phospholipids	Cholesterol: Free	Cholesterol: Total	Cholesterol: Ester*	Glyceride
3	29	None (Vit. B depletion)	—	6/6	7·2	−13	4·9	1·226	4·83	238	151	7·9	8·2	1	79
3A	30	None	—	12/12	10·2	+18	8·1	1·760	20·93	1701	199	15·4	50·0	58	1428
	31	Choline	0·5	12/12	10·2	+18	6·2	1·308	12·63	787	178	13·6	35·0	36	559
	32	Inositol	0·5	12/12	10·2	+20	6·3	1·280	15·78	998	178	12·5	36·5	41	766
3B	33	None	—	9/10	5·4	+ 5	6·1	0·833	32·43	1971	128	13·7	83·3	117	1712
	34	Choline	0·5	9/10	5·8	+18	4·5	0·952	10·95	490	123	11·4	30·7	33	324
	35	Inositol	0·5	9/10	5·3	+ 8	5·4	0·785	28·33	1523	117	12·6	76·4	107	1286
	36	Choline + inositol	0·5 + 0·5	10/10	5·9	+18	4·8	1·042	7·28	350	116	11·9	20·3	14	209
3C	37	None	—	8/10	5·0	+ 9	6·8	1·013	24·47	1670	131	12·3	80·7	115	1411
	38	Choline	0·5	9/10	5·3	+21	5·1	1·086	16·40	769	163	13·5	34·7	36	557
	39	Inositol	0·5	8/10	5·3	+13	6·2	0·912	30·96	1931	118	13·8	87·2	124	1675
	40	Choline + inositol	0·5 + 0·5	8/10	6·0	+22	5·1	1·133	8·71	449	118	10·9	35·9	42	278
3D	41	None	—	11/12	7·2	+ 6	7·4	1·076	35·96	2663	184	16·4	97·0	136	2327
	42	Choline	0·5	12/12	7·1	+ 7	4·6	1·092	8·32	386	173	12·0	43·1	53	148
	43	Inositol	0·5	12/12	7·0	+ 7	6·4	1·051	31·61	2088	173	14·8	106·3	154	1745

compared with the controls, throughout the 16-week period; at 3 weeks 0·3 % inositol was not as effective in reducing steryl esters as was choline and its effectiveness diminished as the experiment was prolonged.

The constancy of the phospholipid values (12·0, 11·3, 12·0, 12·0 % fat-free, dry residue weight for Groups 9, 10, 11 and 12, respectively) is remarkable in view of the large changes in glyceride and cholesteryl esters.

Exp. 2. The results of this experiment (Table 3), in which choline and inositol were fed singly and in combination, at several different levels, for 8 weeks confirm and extend the above-mentioned findings. The first comparisons were made using fat-free test diets (diet 2, groups 13–20) and a similar study was made with diets containing 2 % corn oil (Mazola) plus 1 % cholesterol (diet 2A, groups 21–28).

On the fat-free diets choline fed at the 0·1 % level was almost as effective on all the lipid components as when 0·5 % was provided (compare groups 14 and 15, Table 5). In the presence of cholesterol (groups 22 and 23) the smaller dose of choline was inferior to the larger. The latter brought the liver glycerides nearly to normal and caused an 85 % reduction in cholesteryl esters. Therefore even large amounts of dietary cholesterol (1 %) do not produce a condition as unfavourable for the action of choline as one might be led to expect from statements in the literature (McHenry & Patterson, 1944).

Inositol at 0·1 % dietary level exerted only a very limited lipotropic effect in the rats on the fat-free diet (Group 16) and even less in the presence of the corn oil plus cholesterol (Group 24). Three times as large an intake of inositol had no significantly greater effect on the glycerides and produced only a slightly greater decrease in cholesteryl esters.

In Tables 3 and 5 the effects of various combinations of choline and inositol are also presented. Inositol (0·1 %) added to the diet free from fat and cholesterol (diet 2) did not augment the effect of 0·1 % choline on total lipids or glycerides (compare groups 14 and 18, Table 5). The slight decrease in cholesteryl esters is of questionable significance. When the amount of inositol was increased to 0·3 % a slight but definite effect on both glycerides and steryl esters was noted (groups 14 and 19). Addition of 0·3 % of inositol to a diet already containing 0·5 % choline brought both glycerides and cholesteryl esters within the normal range (groups 15 and 20).

In diets containing fat and cholesterol (groups 21–28), the combined effects of choline and inositol in the amounts mentioned above were qualitatively

Table 5. *Composition of liver lipids*

Basal diet	Group no.	Supplement	%	Duration of exp. (days)	Liver			
					Total lipid	Free cholesterol	Cholesteryl ester	Glyceride
					% fat-free, dry residue weight			
1	1	None	—	21	162	0·83	1·7	148
1A	2	None	—	21	247	1·21	12·4	219
	3	Choline	0·3	21	40	0·93	6·9	22
	4	Inositol	0·3	21	204	1·21	9·9	178
1	5	None	—	56	271	1·10	5·3	252
1A	6	None	—	56	294	1·82	30·4	248
	7	Choline	0·3	56	36	0·96	7·2	14
	8	Inositol	0·3	56	288	1·84	22·2	249
1	9	None	—	112	265	1·15	4·5	247
1A	10	None	—	112	315	2·17	36·7	265
	11	Choline	0·3	112	32	0·90	4·4	15
	12	Inositol	0·3	112	265	1·69	30·2	221
2	13	None	—	56	218	1·17	6·3	195
	14	Choline	0·1	56	52	0·78	1·2	35
	15	Choline	0·5	56	36	0·94	1·0	17
	16	Inositol	0·1	56	168	1·02	2·8	148
	17	Inositol	0·3	56	137	1·18	2·7	115
	18	Choline 0·1 + inositol 0·1		56	53	0·88	0·9	35
	19	Choline 0·1 + inositol 0·3		56	40	0·79	0·6	24
	20	Choline 0·5 + inositol 0·3		56	26	0·93	0·3	9
2A	21	None	—	56	301	1·82	30·6	252
	22	Choline	0·1	56	71	1·11	10·8	40
	23	Choline	0·5	56	38	1·13	4·7	16
	24	Inositol	0·1	56	289	1·68	28·2	239
	25	Inositol	0·3	56	270	1·73	22·7	227
	26	Choline 0·1 + inositol 0·1		56	55	1·15	11·4	24
	27	Choline 0·1 + inositol 0·3		56	45	1·04	5·4	21
	28	Choline 0·5 + inositol 0·3		56	31	1·05	1·6	11
3	29	None (Vit. B depletion)	—	21	19	0·65	0·05	6
3A	30	None	—	7	97	0·88	3·3	81
	31	Choline	0·5	7	60	1·04	2·8	43
	32	Inositol	0·5	7	78	0·98	3·2	60
3B	33	None	—	7	237	1·64	14·1	205
	34	Choline	0·5	7	52	1·20	3·4	31
	35	Inositol	0·5	7	194	1·60	13·7	164
	36	Choline 0·5 + inositol 0·5		7	34	1·14	1·4	20
3C	37	None	—	7	165	1·21	11·4	139
	38	Choline	0·5	7	71	1·25	3·3	51
	39	Inositol	0·5	7	212	1·51	13·6	181
	40	Choline 0·5 + inositol 0·5		7	40	0·97	3·7	25
3D	41	None	—	7	248	1·52	12·6	216
	42	Choline	0·5	7	35	1·10	4·8	14
	43	Inositol	0·5	7	199	1·40	14·6	166

similar to those described in the preceding paragraph. The level of steryl esters in the basal group (21) was increased five-fold by feeding cholesterol. A profound decrease was produced by 0·1% choline. This effect was not augmented by 0·1% inositol but was by 0·3%. The level of steryl esters was lowered still further when choline 0·5% and inositol 0·3% were supplied (group 28) but the value remained above normal. Thus the synergism of choline and inositol can be clearly demonstrated

at certain dosage levels, but here again there is no evidence of any preferential effect of inositol on cholesteryl esters.

Exp. 3. This experiment was designed to compare the relative effectiveness of choline and inositol after a preliminary three-week period of depletion of B-vitamins (the procedure followed by McHenry and his associates). The influence of dietary fat upon the liver lipids of cholesterol-fed animals was also determined. Diet 3A contained 2% cholesterol without any fat, 3B contained an essentially saturated fat (beef dripping 30%), 3C contained the same amount of an unsaturated oil (Mazola) and 3D contained a mixture of these two fats (beef dripping 20%, Mazola 10%).

During the depletion period (diet 3), the food consumption fell from about 8 g. per day to about 3 g. The animals lost weight and several died. The survivors were divided into comparable groups to which the test diets were fed for 1 week only.

In every case choline was more effective than inositol in reducing total liver lipids and cholesteryl esters. The presence of fat in the basal diets (3B, 3C and 3D) increased the total liver lipids greatly. It appears to have caused a slight rise in the free cholesterol (Table 5) and about a four-fold increase in the steryl esters (compare group 30 with 33, 37, 41). The nature of the dietary fat influenced the deposition of glycerides in rats on the basal diets and, to a much smaller extent, that of steryl esters (compare groups 33 and 37). The slightly lower cholesteryl ester value in the rats receiving the corn oil (group 37) as compared with those on beef dripping (group 33) is probably a reflexion of the lower glyceride content of the livers of the 'corn-oil group'. It would appear from the data for groups 34 and 38 that choline is somewhat more effective in reducing glycerides in animals on beef dripping than it is when corn oil is the dietary fat. Inositol produced a small decrease in glycerides in rats on the beef dripping, but seemed to increase the deposition of glycerides in the 'corn-oil group'. This effect, which requires further study, has been noted previously (Beveridge & Lucas, 1945; Handler, 1946). The synergistic effect of choline and inositol was again evident.

When the two fats were present (Basal Diet 3D) inositol had some effect on glycerides, in contrast to the lack of effect on the 'corn oil diet' (3C; cf. groups 37, 39 with 41, 43). However, there was no lipotropic effect of inositol on steryl esters. In the presence of beef dripping there appears to be a partial removal of the blockage of the lipotropic effect of inositol which is observed in the diet containing corn oil alone. An almost completely saturated fat-fraction from beef dripping and a highly unsaturated one from corn oil, have been prepared and their influence upon the lipotropic activity of inositol is being studied.

SUMMARY

1. No waning of the lipotropic effect of choline was observed in rats fed a diet containing 0·5% cholesterol for 16 weeks.

2. Inositol, fed at the same level as choline, was never nearly as active in decreasing glycerides or cholesteryl esters and its effectiveness diminished as the experiment was prolonged.

3. The synergistic lipotropic effect of choline plus inositol was clearly demonstrated in cholesterol-fed rats but inositol did not exert any preferential effect on cholesteryl esters.

4. In experiments, similar to those of McHenry & Patterson (1944) and of Handler (1946) in which the rats were given a preliminary period of deficiency of vitamin B_1, choline was uniformly more effective than inositol in decreasing liver glycerides and cholesteryl esters in cholesterol-fed rats receiving diets with or without fat. These results agree with those reported by Handler (1946).

5. Further evidence that the nature of the dietary fat affects the lipotropic action of inositol has been presented and briefly discussed.

We are indebted to our colleague, Dr C. S. McArthur, whose improvements in the Schoenheimer-Sperry procedure for determining cholesterol made it applicable to tissues containing abnormally large amounts of fat. The expenses of this investigation were defrayed, in part, by a grant from the Banting Research Foundation.

REFERENCES

Best, C. H., Channon, H. J. & Ridout, J. H. (1934). *J. Physiol.* **81**, 409.
Best, C. H., Lucas, C. C., Patterson, J. M. & Ridout, J. H. (1946). *Biochem. J.* **40**, 368.
Best, C. H. & Ridout, J. H. (1933). *J. Physiol.* **78**, 115.
Best, C. H. & Ridout, J. H. (1937). *J. Physiol.* **86**, 343.
Beveridge, J. M. R. & Lucas, C. C. (1945). *J. biol. Chem.* **157**, 311.
Channon, A. & Ludewig, S. (1933). *J. biol. Chem.* **102**, 57.
Handler, P. (1946). *J. biol. Chem.* **162**, 77.
Hinsworth, H. P. & Glynn, L. E. (1941). *Clin. Sci.* **5**, 93.
McHenry, E. W. & Patterson, J. M. (1944). *Physiol. Rev.* **24**, 128.

Inositol Content of Blood Plasma[1]

Sulamith Sonne and Harry Sobotka

From the Division of Chemistry, Laboratories, and the Neurological Service of the Mount Sinai Hospital, New York City

Received February 10, 1947

Introduction

The development of micro bioassay methods for inositol has made it possible to determine this substance in grains and other food stuffs, yeast, animal organs, *etc.*, the content of which is of interest because of the vitamin functions of this cyclic carbohydrate (1). We have adapted the method of Woolley, with the modifications by Atkin (2), to the determination of the inositol content of blood plasma. The procedure consists in a nephelometric evaluation of the growth of a strain (No. 4228) of *Saccharomyces carlsbergensis* after 16–17 hours in a medium supplying all necessary growth factors except inositol. Various details of the method and of the recovery checks, carried out with each test, are described in the Experimental Part.

The range of inositol content in plasma from individual subjects was 0.42–0.76 mg./100 ml. A wider range was found in determinations on pooled plasma from miscellaneous patients: the values were 0.54, 0.65, 0.66, 0.69, 1.00, 1.03, 1.31, 1.32, 1.42, and 1.87 mg./100 ml.

In a number of patients suffering from amyotrophic lateral sclerosis, the values were between 0.37 and 0.67 mg./100 ml. These patients had been selected for inositol studies in view of a report (3) that per oral inositol administration exerted a favorable influence on the therapeutic effects, and thus, by implication, on the absorption and utilization of tocopherol in certain myopathies. No such therapeutic effects could be observed with the use of inositol as an addition to tocopherol in cases of amyotrophic lateral sclerosis; a clinical report will be given by Dr. I. S. Wechsler elsewhere.

One normal subject and four with amyotrophic lateral sclerosis were

[1] Dedicated to Professor Carl Neuberg on his 70th birthday.

given daily 1.50 g. of inositol for several weeks and the plasma level was analyzed at weekly intervals. The table summarizes the results which show moderate irregular increases except for case number 103.

TABLE I

Plasma Inositol Values In Normals And Patients Before And After Inositol Administration

No.		Mg. inositol in 100 ml. plasma			
		Starting level	After administration of 1.5 g. inositol *per diem*		
			for 1 week	for 2 weeks	for 3 weeks
1	Normal	0.54	0.46	0.70	0.93
2	Normal	0.76	—	—	—
3	Normal	0.42	—	—	—
101	Amyotr. Lat. Scler.	0.62	1.04	1.11	—
102	Amyotr. Lat. Scler.	0.47	0.50	0.82	0.75
103	Amyotr. Lat. Scler.	0.67	0.52	0.33	—
104	Amyotr. Lat. Scler.	0.37	0.80	0.57	—
105	Amyotr. Lat. Scler.	0.45	—	—	—

Values obtained by this method in materials, other than blood plasma, have been accepted as true values in spite of the rigorous conditions of hydrolysis. The purpose of this hydrolysis is twofold: to liberate conjugated inositol and to destroy proteins, the presence of which would produce opacity during sterilization. In experiments not reported here, we have attempted to avoid these rigorous conditions for deproteinization of the plasma. In the course of these studies we have reached the conclusion that a considerable portion of the plasma inositol is non-conjugated and ultrafiltrable. Inositol seems to be partly destroyed, or masked, under essentially milder conditions of acidity and time than those used by Woolley and by Atkin. Hence, the plasma inositol values given represent a lower limit and the actual amount of inositol in plasma may be higher. The forms in which inositol occurs in plasma need further investigation.

Experimental

Individual blood specimens were obtained with potassium oxalate in the fasting state in the customary manner. Ten ml. of plasma were hydrolyzed with 100 ml. of 18% HCl for 6 hours and the hydrolyzate filtered through a sintered glass filter.

The filtrate was concentrated *in vacuo*, taken up in 30 ml. of distilled water, treated with a small amount of Norit, and after filtration, the volume was made up to 50 ml. The pH of the acid solution is brought to *ca.* 5.5 by a few drops of 10% NaOH. Usually, duplicate samples of 2, 3, and 4 ml. of this solution are placed in 50 ml. Erlenmeyer flasks, and 5 ml. of the medium are added. Finally, all volumes are made up to 9 ml. with distilled water.

The medium is prepared by combination of the following solutions:

Sugar and salts	500 ml.
Potassium citrate buffer	100 ml.
Ammonium sulfate (15%)	50 ml.
Calcium pantothenate (200 γ/ml.)	25 ml.
Pyridoxine (10 γ/ml.)	50 ml.
Thiamine (10 γ/ml.)	50 ml.
Biotin (1 γ/ml.)	50 ml.
Casein (acid hydrolyzed) 10%	100 ml.
Water	to 1000 ml.

Sugar and Salts Solution. One liter contains 200 g. of C.P. dextrose (anhydrous), 2.2 g. of monopotassium phosphate, 1.7 g. of potassium chloride, 0.5 g. of calcium chloride ($CaCl_2 \cdot 2H_2O$), 0.5 g. of magnesium sulfate, 0.01 g. of ferric chloride, and 0.01 g. of manganese sulfate.

Potassium Citrate Buffer. One liter contains 100 g. of potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$) and 20 g. of citric acid ($H_3C_6H_5O_7 \cdot H_2O$).

Sterilization, inoculation, and incubation are carried out according to Atkin (2) and the final nephelometric reading is performed in a Klett-Summerson photoelectric colorimeter with filter number 66.

In addition to the 6 flasks with aliquots of the unknown, 10 flasks are set up containing standard amounts of 1, 2, 4, 6, and 8γ inositol in duplicate, and also 4 flasks for recovery tests, two of them containing 2 ml. of the unknown + 4γ inositol and two with 3 ml. of the unknown + 2γ inositol. In 26 tests the recoveries at both levels averaged 90% with a standard deviation of 8%.

Acknowledgment

This investigation was supported by a grant from Hoffmann-LaRoche, Inc., to Dr. I. S. Wechsler, Neurologist to the Hospital, and Dr. H. Sobotka, Chemist to the Hospital.

Summary

The application of the nephelometric micro bioassay with *Saccharomyces carlsbergensis* to the determination of inositol in human blood plasma is described.

The range of plasma inositol in normals and certain patients is 0.37–0.76 mg./100 ml. for individual fasting samples. Pooled plasma from miscellaneous patients ranged from 0.54–1.87 mg./100 ml.

Daily ingestion of 1.50 g. of inositol usually produces a moderate rise of the plasma inositol level.

Destruction of some inositol during acid hydrolysis cannot be excluded. The state of inositol in the plasma needs further investigation.

References

1. Woolley, D. W., *J. Biol. Chem.* **140**, 453 (1941).
2. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.* **15**, 141 (1943); **16**, 67 (1944).
3. Milhorat, A. T., and Bartels, W. E., *Science* **101**, 93 (1945).

Klin. Wochschr, 32:567 (1954).

Inositol and Blood Picture

by Helmut Staubach and Helmut Seckfort

Inositol, which belongs to the group of lipotropic substances, has recently been granted, in particular under the influence of American authors[1], more attention in the treatment of fatty liver and arteriosclerosis[2]. Also, in the therapy of malignant tumors, a certain effect has been established with inositol - cancerous tissue should be able to be damaged by inositol[3,4,5].

Thereby is raised the question, whether healthy tissue can also experience injury through inositol application and whether any harm, in particular bone marrow damage, is threatened under the conditions of clinical use of inositol.

Test procedure and results: To clarify this question we have tested (see the particular details of the test procedure[6]) the cellular elements of the peripheral blood for qualitative and quantitative aspects at regular intervals in, collectively, 24 metabolically healthy or mildly ill subjects not only in individual tests (12 cases, each one dose of 2 g inositol intravenously) but also during a longer period of treatment (12 further cases, each 16 days long, 2 g inositol intravenously). In particular, changes of cell structure, which could have indicated damage to the bone marrow, were looked for.

It was shown that the inositol administration produced no structural changes of the blood cells which could indicate an injury to the bone marrow. In addition, there appeared, in contrast to the observations made with choline and methionine application[7], no quantitative change in the blood cell concentration in the periphery.

Summary: Inositol administration to healthy subjects in the above-mentioned doses caused neither qualitative structural nor quantitative concentration changes of the peripheral blood cells.

Literature

[1] Weidlein, A.: A consideration of the physiological importance of inositol, Mellon-Institute, Pittsburgh, Penn. (Dort umfangreiche Literaturangaben.) — [2] Beckmann, R.: Medizinische 1953, 1. — [3] Ritchey, M. G., L. F. Wicks and E. L. Tatum: J. of biol. Chem. 171, 51 (1947). — [4] Laszlo, D., and C. Leuchtenberger: Science (Lancaster, Pa.) 97, 515 (1943). — [5] Hesselbach, M. L., and D. Burk: Rec. Chem. Progr. 5, 37 (1944). — C. A. 39, 1930, 1945. — [6] Staubach, H.: Zur Physiologie und klinischen Bedeutung des Inosit. Inaug.-Diss. Mainz 1954. — [7] Seckfort, H., u. J. Weise: Klin. Wschr. 1954, 294.

J. Biol. Chem. 164:85-91, 1946

BIOLOGICAL CONVERSION OF INOSITOL INTO GLUCOSE*

By MARJORIE R. STETTEN AND DEWITT STETTEN, JR.

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, April 3, 1946)

The fact that the widely distributed natural product, *meso*-inositol, is isomeric with the common hexoses has provoked many attempts to demonstrate the glucogenic nature of inositol in animals. The results of early investigators indicate that when inositol is injected in large doses into animals a maximum of about 50 per cent is excreted unchanged (1). Attempts to relate the fate of the remainder of the inositol to the carbohydrate of the body have been numerous and inconclusive. After administration of inositol to diabetic or phlorhizinized animals no increase in sugar excretion could be shown by Külz (2), while Greenwald and Weiss found a slight increase in the O:N ratio (3). The search for newly formed glycogen in the livers of previously fasted animals given inositol produced negative results in all cases (2, 4, 5). Anderson (6) found no increase in the respiratory quotient after inositol administration. The results of Oppenheimer (7), Embden and Griesbach (8), and Griesbach and Oppenheimer (9) indicate that, unlike glucose, inositol does not bring about lactic acid formation when perfused through surviving livers. On the other hand, Starkenstein (10) showed an increase in lactic acid simultaneous with inositol disappearance in muscle and liver mince, and Mayer (11) isolated lactic acid from the urine of rabbits given inositol subcutaneously. In summarizing these various results Needham (1) finds no proof of the conversion of inositol into glucose in animals.

The recent elucidation of the configuration of *meso*-inositol, which disclosed the possibility that cleavage between a specific pair of carbon atoms would give rise to a molecule of the configuration of *d*-glucose (12), has stimulated a reinvestigation of this problem by more sensitive techniques.

Only if relatively massive amounts of a test substance are rapidly converted into glucose in the animal body will this conversion significantly influence the quantity of glucose in the urine of a diabetic animal or the quantity of glycogen in the liver of a previously fasted animal. If, however, the test substance could be stably labeled with isotope, its conversion to glucose even in small amounts might be detectable by the appearance of

* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation, and the Nutrition Foundation, Inc.

isotope in the glucose excreted or the glycogen formed. Our experience with deuterio-glucose and deuterio-glycogen is in accord with the idea that the hydrogen atoms bound to carbon in these molecules are both chemically and biologically stable (13). It was therefore determined to prepare a sample of *meso*-inositol in which the carbon-bound hydrogens were labeled with deuterium, to administer this material to suitable experimental animals, and to investigate the isotopic composition of the glucose excreted in the urine and of the glycogen deposited in the tissues of these animals.

The preparation of *meso*-inositol by the catalytic hydrogenation of hexahydroxybenzene has been reported by Wieland and Wishart (14). The unique or preponderant formation of one out of nine possible isomers was surprising, but the method seemed ideally suited to the present requirements and was consequently investigated. Noteworthy incongruities in the original description are the fact that at one point the catalyst is referred to as Pd and at another as Pt and the statement that the H_2 uptake was greater than that required by theory. We have made several attempts to repeat this synthesis, investigating both Pt and Pd catalysts at several temperatures and in various solvents, but in no case could a pure product identifiable as *meso*-inositol be isolated.

We therefore sought and found conditions under which the carbon-bound hydrogen atoms of inositol would undergo exchange with deuterium of the solvent. After prolonged shaking of *meso*-inositol in D_2O solution with Pt catalyst at 130–150°, but little decomposition was found to have occurred and there was no evidence of altered configuration. Some but not all preparations of Pt catalyzed an exchange of carbon-bound hydrogen. In aqueous acetic acid no exchange occurred, and the addition of alkali, though increasing the decomposition, did not augment the exchange. Even when exchange did occur, it was notably slow. After 1 week to 1 month of shaking inositol with Pt catalyst in D_2O, deuterium had entered only between 4 and 17 per cent of the carbon-bound positions of inositol.

From such an exchange reaction a sample of inositol was obtained which, after exhaustive removal of deuterium from its hydroxyl positions, contained 1.75 atom per cent of deuterium. This product has been used in the animal experiments to be described. In each case it was administered by intraperitoneal injection in view of the described intestinal disturbance after oral administration of inositol (2, 6). Relatively large amounts were given because of the abundant urinary excretion of injected inositol (5, 10). Two of the test animals were glucosuric rats, one rendered so with phlorhizin, the other with alloxan. The third experiment involved the study of glycogen isolated from previously fasted rats.

2 gm. of deuterio-inositol were injected in divided doses over 7½ hours into a fasted phlorhizinized rat; the urine was collected for 24 hours, during

which time 2 gm. of glucose were excreted. To minimize dilution of any isotope present in this glucose, it was isolated as potassium gluconate; this material contained 0.116 atom per cent D. Whereas it was expected that conversion to potassium gluconate would effect satisfactory separation of urinary glucose from urinary inositol, the possibility was entertained that the isotope apparently present in the gluconate was actually in contaminating inositol. The gluconic acid was therefore converted into its benzimidazole derivative (15) which was predicted to contain $0.116 \times 11/16 = 0.080$ atom per cent D. The finding of 0.072 atom per cent D in this derivative confirms our conclusion that the deuterium was indeed present in the urinary glucose excreted by the rat. As the deuterium content of the body fluids was essentially normal, the source of the deuterio-glucose must have been the deuterio-inositol that had been injected.

Fischer has shown the configurational possibility of *d*-glucose arising from *meso*-inositol by simple ring fission (12). If the deuterio-glucose in the present experiment is pictured as having arisen in this way, the potassium gluconate derived from such glucose molecules should have contained $1.75 \times 12/6 \times 5/11 = 1.59$ atom per cent D. As the potassium gluconate isolated from the urine contained 0.116 atom per cent D, $0.116/1.59 \times 100 = 7$ per cent of the urinary glucose may be accounted for as having arisen from inositol. This is a minimum figure, in that inositol may have been converted into glucose by more elaborate procedures involving the loss of isotope. Since 2 gm. of glucose were excreted during the period of study, at least 2 gm. must have been synthesized, or at least 140 mg. of glucose were formed from the inositol injected. Of the 2 gm. of inositol injected, therefore, a minimum of 7 per cent was utilized for the formation of glucose.

An attempt was made to demonstrate similar formation of glucose from inositol in a rat made diabetic with alloxan. Unfortunately the quantity of urinary glucose in the period following the injection of inositol was small, necessitating its isolation as the osazone and further dilution of the sample prior to its combustion for D analysis. The probable error of the analysis thus became too large to permit the attachment of any significance to the analytical figure (0.02 atom per cent D).

In a third experiment inositol was given to six previously fasted rats which were killed 3 hours thereafter. In accord with the findings of others (2, 4, 5), practically no glycogen was recovered from the livers. Abundant glycogen was found in the muscles, but it proved to contain only 0.020 atom per cent D, which is too low a value for interpretation.

We believe our evidence shows clearly that, at least in the phlorhizinized rat, inositol serves as a precursor for glucose. When compared with other glucogenic substances it is apparently not a very efficient precursor, as is

indicated by the small quantity of glucose that could be shown to arise from the inositol administered. If, after generous administration, only some 140 mg. of inositol are each day converted into glucose by the rat, it is not surprising that its glucogenic nature should have escaped detection by earlier workers. An increment in urinary glucose of this order of magnitude would, in the conventional type of experiment, be scarcely significant.

EXPERIMENTAL

Attempts at Synthesis—A number of attempts were made to synthesize *meso*-inositol from hexahydroxybenzene and related starting materials, according to the conditions of Wieland and Wishart (14) and with variation in the temperature, solvent, and catalyst. Triquinone and hexahydroxybenzene were prepared from hydroquinone by a series of reactions described by Nietzki and Benckiser (16) and Henle (17). Part of the hexahydroxybenzene was purified by repeated recrystallization from concentrated HCl containing $SnCl_2$, and the remainder was converted to hexahydroxybenzene hexaacetate. The hexaacetate could not be reduced at atmospheric pressure in the presence of platinum catalyst (Baker) either at room temperature or at 100° with acetic anhydride, acetic acid, or dioxane as solvent. Reduction of hexahydroxybenzene itself or of triquinone with platinum or palladium catalyst in water at various temperatures between 25° and 100° resulted in hydrogen uptake, often more than required by theory for inositol formation, but no pure product identifiable as *meso*-inositol could be isolated.

Preparation of Deuterio-Inositol by Exchange with Heavy Water—A number of attempts were made to introduce deuterium into carbon-bound positions in inositol by exchange reactions. Carefully purified *meso*-inositol was sealed into bulbs in the presence of platinum catalyst in neutral, acid, or alkaline D_2O, and the bulbs were shaken 1 to 4 weeks at 130–150°. *meso*-Inositol isolated from the solution was analyzed for deuterium. In three out of a total of twelve trials, carbon-bound deuterium was introduced. In all trials in which the platinum oxide catalyst was reduced by bubbling H_2 through the mixture of inositol, catalyst, and solvent, no exchange occurred. Better results were obtained when the catalyst was previously completely reduced by shaking in water under an atmosphere of H_2 and then transferred to the exchange bulb containing the inositol. This has been taken to mean that complete reduction of the catalyst is essential. In such trials no exchange was found to have taken place with 50 per cent acetic acid as solvent, whereas it did occur with distilled water or 0.1 N NaOH. In the best two trial runs in which low concentrations of D_2O were employed, 17.0 per cent of the C-bound hydrogen atoms was exchanged in water and 17.4 per cent was exchanged in dilute alkali in 1 week. When water was used as solvent, *meso*-inositol was recovered in good yields and only slight decomposition was noted, whereas in dilute alkali considerable decomposition took place and less *meso* inositol could be recovered. One attempt was made to introduce deuterium by irradiating a solution of inositol in D_2O with x-rays. When 1 gm. of inositol in 20 cc. of D_2O was irradiated with about 4000 Roentgen units per minute for 1 hour, no stable D was introduced.[1]

The sample of deuterio-inositol used for the biological experiments was prepared by exchange in 99 atom per cent D_2O. 8 gm. of inositol, sealed in a bulb with 25 gm. of D_2O and 1.6 gm. of Pt catalyst, were shaken at 130–150° for 30 days. The solution became light brown in color. The recovered solvent was found to contain 89.5 atom per cent D_2O. Water was repeatedly added to the residue and evaporated away to remove all excess D from the hydroxyl positions of the inositol. Inositol was isolated by precipitation from water with ethanol and recrystallized from aqueous ethanol. 6.41 gm. of a compound were recovered with properties identical with those of the pure *meso*-inositol originally used.

Calculated, C 40.0, H 6.67; found, C 39.95, H 6.44
Starting material, m.p. 224–226°; isolated material, m.p. 225–226°
Mixed m.p. 224–226°

The inositol contained 1.75 atom per cent of D, all of which must have been bound directly to C atoms. The concentration of D in such positions was therefore $1.75 \times 12/6 = 3.50$ atom per cent D, and consequently, $3.50/89.5 \times 100 = 3.9$ per cent of all the carbon-bound hydrogen had undergone exchange.

Inositol Administration to Alloxan-Diabetic Rat—An adult white rat was made diabetic by the injection of 15 mg. of alloxan monohydrate per 100 gm. of body weight. It was found to excrete 6 to 9 gm. of glucose per day when on a diet of Rockland rat pellets. The rat was fasted for a 9 hour preliminary period, after which 2 gm. of deuterio-inositol in 16 cc. of sterile water were injected intraperitoneally in four equal portions at 2 hour intervals. Drinking water was given *ad libitum* but no food was allowed; the urine was collected for 24 hours after the first injection. Since only 78 mg. of glucose were present in this urine, isolation as glucosazone was resorted to, a procedure that necessarily diluted any deuterium that may have been initially present in the glucose, and the 68 mg. of recrystallized osazone obtained had to be diluted further for analysis. The analytical value of 0.02 atom per cent D in this sample was too low for interpretation.

[1] We wish to thank Dr. T. C. Evans of the Department of Radiology for the administration of the x-rays.

Therefore, in the hope of obtaining a larger urinary excretion of glucose, a phlorhizinized rat was next investigated.

Inositol Administration to Phlorhizinized Rat.—An adult white rat, fasted for a preliminary 9 hour period, was made glycosuric by the hypodermic injection of a sesame oil emulsion of 0.01 gm. of phlorhizin per 100 gm. of body weight. 2 gm. of deuterio-inositol in 16 cc. of water were injected intraperitoneally in four doses at intervals of 2½ hours. The urine collected for 24 hours after the first inositol injection was found to contain 2.0 gm. of glucose. A sample of water distilled from the urine contained 0.004 atom per cent D. The urinary sugar was oxidized to gluconic acid and 0.916 gm. of potassium gluconate was isolated. Part of the K gluconate was reprecipitated from water with ethanol and was found to contain 0.116 atom per cent D. The remainder of the K gluconate was converted to its benzimidazole derivative (15). The gluco-benzimidazole, after recrystallization from water, contained 0.072 atom per cent D, as compared with the value of 0.080 predicted on the basis of the D analysis of the K gluconate.

Inositol Administration to Normal Fasted Rats.—After six adult rats had been fasted for 17 hours, each was injected intraperitoneally with 370 mg. of deuterio-inositol in 3 cc. of water. The rats were killed 3 hours later by a blow on the head and glycogen isolated from the total combined livers and from the carcasses (18). Some unabsorbed fluid was noted in the peritoneal cavity. A sample of body water contained 0.014 atom per cent D. There was only a trace of glycogen in the livers. From the eviscerated carcasses 1.13 gm. of glycogen containing 0.020 atom per cent D were obtained.

SUMMARY

meso-Inositol containing an excess of deuterium in its carbon-bound positions has been prepared by platinum-catalyzed exchange with D_2O at elevated temperature.

The administration of deuterio-inositol to a phlorhizinized rat resulted in the appearance of significant concentrations of D in the urinary glucose.

It is concluded that conversion of *meso*-inositol into glucose takes place in the body of the rat.

BIBLIOGRAPHY

1. Needham, J., *Ergeb. Physiol.*, **25**, 1 (1926).
2. Külz, E., *Sitzber. d. Ges. Beförder. ges. Naturwiss. Marburg* (1875), cited by Needham (1).
3. Greenwald, I., and Wells, M. L., *J. Biol. Chem.*, **31**, 1 (1917).
4. von Mering, J., *Arch. ges. Physiol.*, **14**, 274 (1877).
5. Mayer, P., *Biochem. Z.*, **2**, 209 (1907).

6. Anderson, R. J., *J. Biol. Chem.*, **25**, 501 (1916).
7. Oppenheimer, S., *Biochem. Z.*, **45**, 30 (1912).
8. Embden, G., and Griesbach, W., *Z. physiol. Chem.*, **91**, 251 (1914).
9. Griesbach, W., and Oppenheimer, S., *Biochem. Z.*, **55**, 323 (1913).
10. Starkenstein, E., *Z. exp. Path. u. Therap.*, **5**, 378 (1908).
11. Mayer, P., *Biochem. Z.*, **9**, 533 (1908).
12. Fischer, H. O. L., *Hoppe-Seyler's*, **99**, 156 (1914–15).
13. Stetten, D., Jr., *J. Biol. Chem.*, **159**, 123 (1945).
14. Wieland, H., and Weichert, R., *Ber. chem. Ges.*, **47**, 2082 (1914).
15. Moore, S., and Link, K. P., *J. Biol. Chem.*, **132**, 293 (1940).
16. Nietzki, R., and Benckiser, T., *Ber. chem. Ges.*, **18**, 499 (1885).
17. Heule, F., *Ann. Chem.*, **359**, 329 (1908).
18. Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **155**, 231 (1944).

DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION

XXXI. FURTHER STUDIES ON THE ROLE OF P AMINOBENZOIC ACID AND INOSITOL IN LACTATION AND GROWTH OF THE ALBINO RAT[1]

BARNETT SURE

Department of Agricultural Chemistry, University of Arkansas, Fayetteville

(Received for publication February 27, 1943)

In 1941 it was shown that as a source of the vitamin B complex, adequate daily doses of pure crystalline thiamine, riboflavin, pyridoxine, choline chloride, calcium pantothenate and the "W" factor from liver extracts resulted in complete failure in lactation of the albino rat. The missing factor, tentatively designated as "Bx", was found in rice polishings, defatted wheat embryo, dried grass, and brewers' yeast, but most abundantly in liver and rice bran extracts (Sure, '41).

Following the suggestion of Ansbacher ('41) that p-aminobenzoic acid may be a component of the vitamin B complex, experiments were carried out to determine if this aromatic acid is a constituent of or identical with the "Bx" factor. Definite responses to p-aminobenzoic acid were obtained in lactation (Sure, '41); these have been now confirmed with numerous additional experiments, the results of which are presented in this communication.

The report of Woolley ('41) that inositol is an essential factor for growth of the mouse, as well as the claim of Pavcek and Baum ('41) that inositol is a growth-promoting substance for the rat, warranted its trial in lactation. However, the preliminary positive responses in the case of only a few litters of nursing young were not confirmed by much more extensive studies in which inositol was used throughout the growth, reproduction, and lactation periods. The results of this investigation are presented in tables 1 and 2.

[1] Research paper no. 773 Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station. Aided by a grant from the Committee on Scientific Research of the American Medical Association.

TABLE 1

Composition of Sure's salts no. 2.

	gm.		gm.
NaCl	337.0	$MnSO_4$ (anhydrous)	8.0
K_2HPO_4 (anhydrous)	600.0	$ZnCl_2$	0.5
$CaHPO_4.2H_2O$	190.0	$CuSO_4$ (anhydrous)	0.4
$MgSO_4$ (anhydrous)	99.0	$Al_2(SO_4)_3K_2SO_4$	0.4
$CaCO_3$	600.0	NaF	0.5
Ferric citrate (powdered)	55.0	Cobalt chloride	0.5
KI	1.6	Sodium tetra borate (anhydrous) ($Na_2B_4O_7$)	0.5

Before the new series of lactation experiments were initiated, a modification in the salt mixture was made, increasing the $MnSO_4$ from 7.0 to 8.0 gm., and adding cobalt and boron as trace elements, as a factor of safety, to insure optimum mineral requirements, although boron has not yet been shown to be essential for the animal organism. The composition of the new salt mixture, designated as Sure's salts no. 2, is given in table 1. To further insure adequate mineral intake, the new salt mixture was fed at a 5 instead of a 4% level. Because of the high moisture content and the lack of absolute purity, the blood fibrin rations were fed at a 25% plane of intake, which was the equivalent of 20% protein, calculated from the total nitrogen content.

It was realized that the "Bx" factor may be a composite of several unknown components of the vitamin B complex. Therefore, in order to secure a response to a single chemical substance such as p-aminobenzoic acid, small amounts of natural foods or extracts therefrom, were incorporated in some of the rations, i.e., 2% dried grass in ration 13, 2% dried liver in ration 14, and 1% liver concentrate in ration 12. Rations 12, 13, and 14 also contained 0.1% ascorbic acid. Numerous other

Jour. Nutrition 26(3):275-283, 1943

TABLE 2

The role of p-aminobenzoic acid and inositol in lactation.

RATION NO.	GROUP NO.	SOURCE OF PROTEIN	SOURCE OF CARBOHYDRATES	SPECIAL ADDITIONS	PARA-AMINOBENZOIC ACID OR INOSITOL ADDITIONS	NO. OF FEMALES MATED	NO. OF LITTERS	TOTAL YOUNG BORN	YOUNG GIVEN TO RAT	WEANS REARED	PER CENT WEANS REARED
13	1	{Casein, 10% Fibrin, 10%	Dextrin	Dried greens, 2%	Control	5	6	64	36	6	16.7
13		"	Dextrin	"	inos.[1]	5	6	56	35	6	17
13		"	Dextrin	"	p.a.b., inos.[1]	5	8	65	41	16	39.0
12	2	{Casein, 10% Fibrin, 10%	Dextrin	Liver concentrate, 1%	Control	5	10	127	60	20	33.3
12		"	Dextrin	"	inos.[1]	5	11	96	63	10	15.9
12		"	Dextrin	"	p.a.b., inos.[1]	5	9	106	54	33	61.1
14	3	{Casein, 10% Fibrin, 10%	Dextrin	Dried liver, 2%	Control	5	9	92	40	23	57.5
14		"	Dextrin	"	inos.[1]	5	9	88	53	5	9.4
14		"	Dextrin	"	p.a.b., inos.[1]	5	11	103	60	51	85.0
8	4	Fibrin 25%	Dextrin	Yeast nucleic acid, 0.2%	Control	5	10	97	59	18	31.5
9		Fibrin, 25%	Dextrin	"	inos., 0.2%	5	6	44	23	5	21.7
10		Fibrin, 25%	Dextrin	"	p.a.b., 0.2%	5	6	67	36	18	50.0
11		Fibrin, 25%	Dextrin	"	p.a.b., 0.2% inos., 0.2%	5	6	50	16	5	31.2
7	5	Casein, 22.5%	Cerelose		Control	10	13	117	77	17	22.1
21	5	Fibrin, 25%	Cerelose		Control	15	32	340	216	87	40.3
3		Fibrin, 25%	Cerelose	Polished rice, 30%		5	11	96	66	44	66.6
4		Fibrin, 25%	Cerelose	"	inos., 0.2%	5	12	112	67	21	31.3
5		Fibrin, 25%	Cerelose	"	p.a.b., 0.2%	5	11	112	63	62	98.4
6		Fibrin, 25%	Cerelose	"	inos., 0.2% p.a.b., 0.2%	5	10	108	60	53	88.3
1		Fibrin, 25%	Dextrin		Control	15	21	193	119	33	27.7

[1] Administered separately from the ration in doses outlined in the text.

[2] p.a.b. = p-aminobenzoic acid; inos. = inositol.

experiments, not included here, showed no special benefits in lactation from ascorbic acid additions. All the rations contained small amounts of nicotinic acid. The cystine additions to rations containing casein were made only during the reproduction and lactation periods. Two sources of carbohydrates were used, a commercial dextrose [2], and dextrin, the latter prepared in the laboratory by cooking cornstarch for 3 to 4 hours at 15 to 17 pounds pressure.

Adequate amounts of the various components of the vitamin B complex were administered separately from the rations in doses outlined in a previous publication (Sure, '41). Vitamins A and D were furnished by 3 drops of halibut liver oil to each animal once weekly until mating, and 3 drops three times weekly during pregnancy and lactation. Vitamin E was provided by wheat germ oil in the rations.

COMPOSITION OF RATIONS

The percentage compositions of diets 1 and 2 were as follows: blood fibrin [3] 25, butterfat 10, carbohydrate 56.95, Sure's salts (no. 2) [4] 5, wheat germ oil 3, and nicotinic acid 0.05. These diets differed merely in that diet 1 had dextrin as the carbohydrate whereas diet 2 had dextrose.[5] Diet 3 was the same as diet 2 except that it contained 30% polished rice with the dextrose reduced accordingly. Diet 4 differed from diet 3 in that the former had 0.2% inositol with a further corresponding reduction in the dextrose. Diet 5 was the same as diet 4 except that no inositol was present but, instead, 0.1% p-aminobenzoic acid; and diet 6 contained both the 0.2% inositol and 0.1% p-aminobenzoic acid, all such additions being at the expense of the dextrose. The p-aminobenzoic acid was increased to 0.2% during the reproduction and lactation periods.

In ration 7 the variable of interest was a commercial vitamin-free casein [6], this constituting 22.5% with a corresponding change in the carbohydrate component of the ration.

[2] Cerelose.

[3] Thoroughly extracted with 95% ethanol.

[4] Table 1.

[5] See footnote 2.

[6] Supplied by the S.M.A. Corporation.

Rations 8, 9, 10 and 11 represented test of diet 1 with 0.2% yeast nucleic acid and the further addition of either 0.2% inositol or 0.1% p-aminobenzoic acid or both.

Diets 12, 13, and 14 differed from diet 1 in the following respects: instead of 25% fibrin being used as the protein, a combination of 10% fibrin, 10% washed casein[7] and 0.2% cystine was employed. All three diets contained 0.1% ascorbic acid. Diet 12 had 1% liver concentrate, diet 13 had 2% dried grass, and diet 14 contained 2% dried liver, all of these changes from diet 1 being at the expense of the carbohydrate component, which was dextrin.

EXPERIMENTAL

This study was conducted with five groups of animals. Groups 1, 2, and 3 received inositol and p-aminobenzoic acid separately from the ration. Inositol was administered in 10 to 15 mg. daily doses per animal until mating, and in 20 mg. daily doses during the reproduction and lactation periods. p-Aminobenzoic acid was fed in 3 mg. daily doses per animal during the first 4 weeks, followed by 7.5 mg. daily doses until breeding, and 15 mg. daily doses during pregnancy and lactation. Groups 4 and 5 received inositol and p-aminobenzoic acid in the rations, as shown in table 2.

Groups 1, 2, and 3. It will be noted from table 2 that inositol produced a very marked harmful effect on lactation when present in rations containing 2% dried grass, 1% liver concentrate, and 2% dried liver, respectively. On the other hand, p-aminobenzoic acid exerted a most pronounced protective action against inositol injury, as evidenced from efficiency in rearing of young. On ration 13 there was a change from 0 to 39%; on ration 12, from 15.9 to 61.0%; and on ration 14, from 9.4 to 85%.

Group 4. Because of the claim of Nakahara, Inukai, and Ugami ('41) that yeast nucleic acid may be a component of their L_2 factor, the rations in this group of experiments were fortified with 0.2% of such product.[8] In this experimental set-

[7] Thoroughly extracted with chloroform and dilute ethanol.

[8] Obtained from Lactonase Products Co., Rochester, New York.

up p-aminobenzoic acid was added alone (diet 10) as well as in combination with inositol (diet 11). The injurious effect of inositol is again apparent. The supplementary effect of p-aminobenzoic acid is most marked. However, while there was protection against inositol injury in the presence of the latter, p-aminobenzoic acid, in group 4, did not show its supplementary role.

Group 5. The most surprising results were secured in this group of experiments. Previously, on the basal ration, in the absence of natural foods or the "Bx" factor, the lactation efficiency was about 6% when the protein was furnished by either 20% blood fibrin or by 10% casein and 10% blood fibrin, fortified with 0.2% cystine; also when dextrin was the source of carbohydrates, and when 4% of Sure's salts no. 1 (Sure, '41) provided all the minerals. Ration 2, containing 25% blood fibrin, dextrose as the source of carbohydrates, and 5% of Sure's salts no. 2, allowed 40% success in rearing of young. In other words, this radical change in the basal ration resulted in an increase of efficiency of 34% in lactation. The first experiments with this new basal ration were conducted with five females. This was later extended and results were obtained on fifteen mothers which bore thirty-two litters of 340 young. Another group was then started with fifteen females on the same type of ration but dextrin replaced glucose. While it is not possible at this time to make a fair comparison between the efficiency of glucose versus dextrin for lactation, since the mothers on the glucose diets had second litters while those on dextrin rations had only first litters, still the indications are already apparent that glucose is superior to dextrin for lactation, perhaps by allowing greater efficiency of bacterial synthesis of biotin, folic acid or unknown components of the vitamin B complex (Martin, '42b).

A comparison for lactation was also made between pure vitamin-free casein (diet 7) and blood fibrin (diet 2) using glucose and 5% of Sure's salts no. 2. From the results given in table 2 it is evident that casein, even when fortified with cystine, proved an inferior protein to blood fibrin for lactation.

The substitution of 30% glucose with an equivalent amount of highly milled rice (polished) increased the efficiency of rearing of young from 40.3 to 63.6%. Evidently the polished rice furnished an unknown factor or factors of the vitamin B complex essential for lactation. The addition of 0.2% inositol reduced lactation efficiency by about 50%. However, the addition of 0.2% p-aminobenzoic acid increased lactation efficiency to practically complete success. Also, addition of 0.2% p-aminobenzoic acid to a ration containing 0.2% inositol not only counteracted the harmful effects of the latter but showed almost all of its supplementary effect.

Growth experiments with p-aminobenzoic acid and inositol

The influence of p-aminobenzoic acid on growth of weaned albino rats, with initial weights of 34 to 46 gm., was studied by the paired feeding method on nine pairs of females and twelve pairs of males for 100 to 120 days. This study was carried out on a purified synthetic diet containing 18% vitamin-free casein. The daily dose of the aromatic acid was 3 mg. to the positive control animals. The daily doses per animal of the rest of the components of the vitamin B complex were as follows: 20 µg. thiamine, 20 µg. riboflavin, 20 µg. pyridoxine, 6 mg. choline chloride, 0.5 mg. nicotinic acid, 100 µg. calcium pantothenate, and 10 mg. inositol. The growth was excellent but there was no additional growth produced by the administration of p-aminobenzoic acid. Similarly, inositol addition, in the presence of p-aminobenzoic acid produced no growth responses in seven pairs of females and eleven pairs of males during a period of about 4 months, but there was no injurious effect on growth precipitated by inositol, as has been observed in lactation. The daily dose of inositol to the positive controls was 10 mg. These results are in harmony with those of Unna, Richards and Sampson ('41), Emerson ('41), and Martin ('42a). The reason for p-aminobenzoic acid being essential for lactation and not for growth of the weaned rat is not clear at present, unless there is a difference in the efficiency of synthesis of this substance between very small

nursing young and weaned rats whose capacity for synthesis has been established at that age and weight.

It is of interest to note that Martin ('42a), working with growing animals and the author with lactating animals have noticed dietary disturbances following inositol addition to the vitamin B complex mixture, which however, were counteracted by administration of p-aminobenzoic acid. Martin offers the hypothesis that inositol stimulates the growth of microorganisms which utilized and destroy some member of the vitamin B complex, known or unknown, thus precipitating a deficiency of that factor. It is also possible, however, that excess inositol may interfere with the synthesis of biotin, folic acid, or some other unknown member of the B complex. The response of p-aminobenzoic acid could then be interpreted as supplying a factor essential for synthesis of such substances as biotin and folic acid rather than for neutralization of a toxin (Martin, 42b). The syndrome produced by Martin following addition of p-aminobenzoic acid to the Rockland strain black rat was not confirmed by the author's observations on the weaned or lactating Wistar strain albino rat.

SUMMARY

p-Aminobenzoic acid has a markedly favorable influence on lactation of the albino rat. On the other hand, inositol has a pronounced injurious influence on lactation, which is counteracted by p-aminobenzoic acid.

LITERATURE CITED

ANSBACHER, S. 1941 p-Aminobenzoic acid, a vitamin. Science, vol. 93, pp. 164–165.

EMERSON, G. A. 1941 Failure to cure or prevent greying of rats with p-aminobenzoic acid. Proc. Soc. Exp. Biol. and Med., vol. 47, pp. 448–449.

MARTIN, G. J. 1942 a The interrelationship of p-aminobenzoic acid and inositol. Am. J. Physiol., vol. 136, pp. 124–127.

——— 1942 b "Folic acid" in nutritional achromotrichia. Proc. Soc. Exp. Biol. and Med., vol. 51, pp. 353–355.

NAKAHARA, W., F. INUKAI AND S. UGAMI 1941 Activity of yeast nucleic acid as vitamin L_2. Proc. Imp. Acad. Tokyo, vol. 17, pp. 266–268.

PAYCHE, P. L., AND H. M. BAUM 1941 Inositol and spectacled eye in rats. Science, vol. 93, p. 502.

SURE, B. 1941 Dietary requirements for fertility and lactation. XXIX. The existence of a new dietary factor essential for lactation. J. Nutrition, vol. 22, pp. 499–514.

WOOLLEY, D. W. 1941 Identification of the mouse antialopecia factor. J. Biol. Chem., vol. 139, pp. 29–35.

UNNA, K., G. V. RICHARDS AND W. S. SAMPSON 1941 Studies on nutritional achromotrichia in rats. J. Nutrition, vol. 22, pp. 553–563.

Jour. D. D.
February, 1943

Volume 10
Number 2

Clinical Experiments With Riboflavin, Inositol and Calcium Pantothenate

By

MARTIN G. VORHAUS, M.D., MICHAEL L. GOMPERTZ, M.D.

and

AARON FEDER, M.D.

NEW YORK, NEW YORK

DURING the past few years, the unsolved problems of nutritional deficiency states have been vigorously and successfully attacked by the biochemists and other investigators in related fields of science. New vitamins have been isolated, synthesized and administered to animals under varying experimental conditions. Although the preliminary reports of these studies seem confusing and at times contradictory, they are of great interest to the clinician. They challenge him to clarify and correlate this new knowledge and to evaluate its use in human deficiency states. It is the purpose of this report to describe such clinical investigations.

The therapeutic value of riboflavin in cases of cheilitis led us to study its effect in the treatment of decubitus with ulceration (7). Cases were accepted irrespective of the severity of the associated conditions. No case showing any clinical improvement on ordinary ward care was included in this series. Six cases are described in detail of which three were controlled rigidly. In these three cases no other vitamin supplement was given and no local treatment of the ulcer was permitted. In the other three cases the original disease required additional treatment which may be interpreted as having had some influence upon the decubitus. This additional treatment is described in detail.

CASE REPORTS

Case 1. S. G., white male, aged 69, diagnosis—inoperable carcinoma of the sigmoid. Palliative transverse colostomy was performed on 1-9-41 and shortly thereafter a decubital ulceration directly over the sacrum was noted. The ulcer progressed steadily and on 1-22-41 measured 6.4 x 5.2 cms. The base was pale and unhealthy but the surrounding tissues seemed normal. The usual oral dose of 5 mgms. of riboflavin was started at that time and continued daily thereafter. After four days of therapy the ulcer measured 5.7 x ⅜ cm. and from then on continued improvement was seen, until on 2-19-41 (after 28 days of riboflavin therapy), the ulcer was completely healed. Three days later the patient was transferred to another hospital with no evidence of the previous sacral ulcer.

Case 2. A. J., white female, aged 66, diagnosis—arteriosclerotic heart disease with decompensation, bundle branch block. Admitted 4-22-40. Decubital ulcers which became progressively worse were observed on each buttock. On 5-2-40 an ulceration measuring 2.5 x ½ cm. was noted on the left buttock, and on the right buttock a smaller ulcer measuring 1.2 x 0.5 cm. On this day the daily oral dose of 5 mgms. of riboflavin was begun. In three days beginning improvement was noted and by 5-8-40 (five days later) the left buttock ulcer measured only 1 cm. in length and the right ulcer was almost healed. Gradual healing continued until by 5-24-40, after three weeks of treatment, both ulcers were entirely healed and the skin of the buttocks appeared normal. This patient was observed until discharge on 6-2-40 and no evidence of the previous decubitus was noted.

Case 3. M. R., white male, aged 60, diagnosis—arteriosclerotic gangrene of left lower extremity. Enormous decubital ulceration, shaped like an inverted triangle and involving about one-third of lower back was seen. Skin and subcutaneous tissue were missing over entire ulcerated area which varied in depth from one to three cms. Cheilitis at angles of the lips and outer canthi of both eyes were noted. On 4-8-40, nine days after admission, 5 mgms. of riboflavin daily were given orally. Patient was under observation until death on 4-15-40. During these seven days the purulent discharge from the ulcer ceased. Necrosis diminished and small areas of granulation were observed. At the time of death the ulcer was found to be reduced more than one-third in area and the cheilitis of the lips and canthi of the eyes had disappeared.

Case 4. J. C., white male, aged 44, diagnosis—thrombophlebitis of inferior vena cava with marked brawny induration of entire body below the costal margins complicated by suppuration in left thigh, probably secondary to the deep thrombophlebitis. On 2-24-40 beginnng bed sore in sacral region noted. On 3-5-40 incision and drainage of abscess in left thigh. Progressive development of bed sore which became larger and deeper. On 3-20-40 this round ulcer over the sacrum measured 2.25 cms. in diameter; its edge was beveled and its base appeared unhealthy. Five mgms. of riboflavin by mouth started and continued daily. Ferrous sulfate and a whole B complex preparation were also given. In three days the ulcer began to improve. On 3-25-40 it had decreased to 1.75 cms., on 3-29-40 further decrease to 1 cm. was noted. On 4-2-40 (thirteen days later) the ulcer was completely healed. At the time of the patient's discharge from the hospital (4-23-40) the sacral area appeared healthy and had remained free of ulceration.

Case 5. G. S., white female, aged 58, diagnosis—oblique fracture of left femur. Admitted 4-12-40. On 4-30-40 pressure erosion was first noted in sacral region and over left lateral epicondylar region of the thigh. During the next ten days these lesions became progressively worse. At that time the sacral lesion measured 4 x 2 cms. and the ulcer over the left thigh 3 x 2 cms. The surrounding skin was healthy. The daily oral administration of 5 mgms. of riboflavin was started on 5-10-40. From 5-10-40 to 5-13-40 one tablet of Brewers yeast was given three times a day by error but was discontinued on the latter date. During the period of riboflavin administration, estrone, which had been given for some time, was continued. By 5-17-40 the sacral ulcer was almost entirely healed and the thigh ulcer was reduced to a size of 2.5 x 1.3 cms. Seven days later (5-24-40) the ulcer of the thigh was completely healed. Riboflavin was reduced to 2 mgms. daily. This patient was observed until discharge on 7-30-40 and the skin in the affected areas remained healthy.

*Read before the American Gastro-Enterological Association at Atlantic City on June 9, 1942.
From the Medical Service of the Hospital for Joint Diseases.
The Inositol and the Calcium Pantothenate were supplied by the Abbott Laboratories.

Case 6. S. C., white male, aged 15, diagnosis — congestive heart disease with circulatory collapse, pulmonary edema followed by pneumonia, interlobar pulmonary effusion and thoracentasis. Admitted 11-21-40. On 12-6-40 an area of gangrene was noted over the sacrum. On 12-11-40 the decubital ulcer measured 5.1 x 3.2 cms. Its central portion had a depth of 2 cms. and the base of the ulcer at this portion was covered with foul-smelling necrotic tissue. The oral administration of 5 mgms. of riboflavin was begun on 12-11-40 and continued daily thereafter. Six days later this patient was placed on a high vitamin, high calorie diet. In six days the foul center slough had become clean and by 12-19-40 (eight days later), the ulcer had decreased to 3 x 3.2 cms. in size. On 12-20-40 sulfathiazole 0.5 G. t.i.d. was given for two weeks, and on 1-11-41 a transfusion of 250 cc. was also given. By 1-15-40 the ulcer measured 1.25 cms. in diameter and tissue loss was only superficial. Improvement continued, with complete healing of the lesion noted on 2-3-41 (seven weeks later), but sulfathiazole had been resumed on 2-2-41 in doses of 1 G. Q 4h. and was continued until 2-9-41. The following day he received an additional transfusion of 500 cc. This patient remained under observation until discharge from the hospital on 3-6-41, at which time the sacral area was healthy.

DISCUSSION

Six cases of decubital ulceration were treated by the daily oral administration of 5 mgms. of riboflavin. Five of these cases showed complete healing of the ulcers in from seven to thirty-four days of riboflavin therapy. The sixth case died after seven days of treatment with riboflavin and at death the decubital ulceration was reduced in size and showed evidence of healing.

Since improvement in these six cases was noted within a few days after the institution of riboflavin, it would seem likely that the healing of these ulcers was due to the vitamin therapy. The continued and progressive development of these lesions up to the day on which riboflavin was first given should be emphasized. It is unlikely that the improvement in the local lesion could have been an expression of a generalized systemic improvement. On the contrary, the general condition of Case 2 grew progressively worse and ended in death; Case 5 had an inoperable carcinoma of the sigmoid.

It is not to be inferred from these observations that decubital ulceration is a clinical manifestation of ariboflavinosis, nor are these results to be interpreted as evidence that riboflavin is a specific in the treatment of bed sores. It is interesting to recall that early in our clinical experiments with riboflavin many observers believed that cheilitis was a clinical state characteristic of riboflavin deficiency. Subsequent studies have been reported in which the administration of pyridoxine in cases of cheilitis has been followed by the disappearance of these lesions. Furthermore, cases of cheilitis which have been resistent to riboflavin therapy have shown complete healing when pyridoxine was given (5).

It is extremely likely that other members of the B complex as well as riboflavin may exert a favorable effect upon decubital ulceration. Quite probably the addition of the entire B complex to the riboflavin administered might have resulted in a more rapid improvement in the local lesions of these six cases. Such clinical possibilities are in keeping with our increasing knowledge of vitamin therapy.

Bed sores are generally slow-healing, indolent lesions and their rapid response to riboflavin therapy gives rise to speculation as to the mechanism involved. Riboflavin is an essential factor in nutrition and is of great importance to the oxidation enzymes of the body. It is believed to be a basic constituent of the xanthine oxidase and thus establishes a closer link between vitamins and tissue enzymes (1).

The lesions of the skin and subcutaneous tissue in cheilitis have a parallel in miniature to the tissue changes seen at times in decubital ulceration, and the clinical response in both of these lesions to the administration of riboflavin serves to emphasize this parallelism. Further speculation seems to relate the various members of the B complex to tissues of original ectodermal origin; thiamin to nerve tissue, nicotinic acid to skin and mucous membrane, riboflavin and probably pyridoxine to skin and subcutaneous tissue. Now that inositol and pantothenic acid are available it seems logical to conjecture whether these two newer members of the B complex are not also possessed of an influence on epithelium or similar tissues.

Although inositol was first isolated in 1850 (19), it was not believed to have an essential role in nutrition until Woolley in 1940 identified it as the mouse antialopecia factor (13). The alopecia produced in mice by a deficient diet was not influenced by biotin or p-aminobenzoic acid. This diet contained adequate pantothenic acid, in addition to other members of the B complex. However, when inositol or some of its derivatives were added to the diet the alopecia disappeared (12).

Pure inositol was made available to us for study and it seemed logical to explore its possible value in diseases of the skin and hair. On the basis of present concepts the daily adult requirement of the human being is about one gram. Several volunteers were asked to take this amount daily and no untoward effects were observed.

In a recent communication, studies on animals suggested that inositol appears to stimulate intestinal peristalsis (6). This question has been followed clinically in twenty patients taking from one to two grams of inositol daily. In not one of these cases was any change in the normal bowel habits noted, even though many of them had mild to moderate constipation.

Gavin and McHenry found a similarity between the action of inositol and lipocaic in preventing the "biotin" type of fatty liver (3). With this in mind inositol was administered to a 58 year-old female diabetic with a blood cholesterol of 398 mgms. This patient also had a bilateral symmetrical thickened eruption of the lower extremities and back. After the patient had received 2 grams of inositol daily for three weeks the eruption had cleared. The cholesterol was 313 mgms. several days after discontinuing the inositol. Further studies are in progress and will be reported.

Cases of alopecia have been selected for study and daily doses of from one to two grams of inositol have been administered to these patients for several weeks and in some instances for over two months. Definite conclusions are not possible at this time, but on the basis of our early observations there are not sufficient changes to indicate that inositol is the human antialopecia factor.

With the aid of our associates in the field of dermatology a systematic survey is at present being made of

the possible value of inositol in a variety of skin diseases hitherto resistant to treatment. These detailed studies will be reported later, but our preliminary observations seem to indicate that inositol may have a value in some skin diseases. Two examples of such a response are described in detail.

An 84 year-old female suffering from an extensive generalized pruritic eruption with thickening and desquamation was treated by a dermatologist for four months. The eruption failed to respond to a variety of local medication, X-ray and Vitamin A orally. She volunteered for inositol therapy. After all other treatment had been stopped for three weeks and no change in the eruption had occurred, she was given 0.5 gram tablets of inositol orally, twice a day. In ten days there was a lessening of the pruritus, in two weeks a modification of the eruption, and at the end of five weeks the eruption had almost entirely disappeared.

A 51 year-old male diabetic had a pruritic thick scaly symmetrical eruption involving both ankles and the inner aspect of both feet. Smears and cultures for fungi were negative, and treatment for two years by various dermatologists had not modified the eruption. He volunteered for inositol and was placed on one gram daily by mouth. Within two weeks there was a reduction in the amount of the eruption and at the end of nine weeks the eruption had almost entirely disappeared. Medication was then discontinued in order to evaluate clearly the effect of the inositol upon the eruption. Within two weeks the eruption again was manifest and at the end of two months had developed to its original extent and severity. Inositol has been resumed recently with its original beneficial effect.

Since the chemical identification of pantothenic acid and its synthesis two years ago (9, 11, 10), it has been confirmed over and over again that pantothenic acid is one of the factors primarily responsible for the prevention of nutritional achromotrichia in animals (8, 2, 4).

Recent studies in animal experimentation indicate that inositol and calcium pantothenate exert a definite influence on tissues of ectodermal origin. Woolley has emphasized the relationship between inositol and pantothenic acid, since the quantity of pantothenic acid fed influenced his animal cases of alopecia (14, 15). In some instances, with inclusion of large amounts of calcium pantothenate in the diet, no alopecia developed, even though there was a marked absence of inositol in the rations fed. The explanation for this tends to emphasize more emphatically the inter-relationship of vitamins of the B complex. It has recently been reported that mice are able to synthesize inositol, if sufficiently high levels of pantothenic acid are fed, by means of intestinal micro-organisms (17). These reports are so very recent that they are mentioned here only to show the trend in animal experimentation. Thus far, no human clinical research in this direction has been reported. Woolley has shown that mice fed on a low pantothenic acid diet developed signs of inositol deficiency. When these animals are given further supplements of inositol by mouth their signs of inositol deficiency continue unabated. Woolley raises the question as to whether there is interference of inositol absorption or destruction of the ingested inositol. In this connection Martin has varied these experiments in an attempt to answer this question. When his mice on a low pantothenic acid diet developed signs of inositol deficiency he injected inositol intramuscularly and observed the disappearance of the signs of inositol deprivation (18).

Calcium pantothenate was made available for study. One hundred mgms. of pantothenic acid were given twice a week by intramuscular injections to three volunteers for six weeks. No effect upon the gray hair present was noted. In one case of alopecia totalis (a 54 year-old white male) one gram of inositol was given daily by mouth and 100 mgms. of calcium pantothenate were injected intramuscularly twice a week for six weeks. At the end of this test period no change was noted and the experiment was discontinued.

A 45 year-old white male with alopecia areata for three years volunteered for treatment. Before treatment white hair had regrown in some of the areas of alopecia. 655 mgms. of calcium pantothenate were given by intramuscular injection during ten weeks in varying amounts, and at the end of this time there was an unmistakable new growth of black hair in a central white streak. In the next two months 950 mgms. more of calcium pantothenate were given. At the end of that time no new growth was noted, although the previous old black hair was about one inch long. The treatment was stopped for two months and at the end of that time there was no change in the hair. In the next two months 42 grams of inositol were given in divided doses by mouth, and no change was noted. Then calcium pantothenate was again given by injection in doses of 500 mgms. intramuscularly twice a week. Twelve injections (a total of 6000 mgms.) were given and a slight new growth of black hair was observed. During all this time no pigmentation was noted in the areas of white hair present in this patient. Further studies are in progress and will be reported.

DISCUSSION

Although inositol has been known for many years and used extensively in animal experiments by Woolley in the past two years, no reports of its use in humans have appeared. Since we have reason to believe that this is the first time pure inositol has been given to human subjects, observations were made on doses of inositol that approached physiologic amounts. From analyses of the human diet it is assumed that the daily requirement of inositol is about one gram. All our observations were made in cases receiving one or two grams daily and the inositol was given in the form of 0.5 G. tablets in divided doses, irrespective of meals.

Our preliminary observations indicate that in these doses there is no apparent harmful effect of inositol in healthy subjects. Furthermore, the bowel habits of our patients were not modified by this amount of inositol and therefore in these doses there is no clinical confirmation of the previous report that inositol stimulates intestinal peristalsis.

The results of animal experiments with inositol present two outstanding facts—firstly, that the signs of inositol deficiency in animals seem to be mainly changes in the skin and hair; and, secondly, that inositol is used by animals to make more complex tissue substances. One such substance has been identified as phosphatide, found in the brain and nerve tissue; it appears to be a combination in some complex

form of inositol and phosphates (16). This combination is lipid soluble in contradistinction to the water soluble compound in muscle which is made up of inositol and other as yet unidentified substances (18).

After we were convinced that inositol has no harmful effect in our doses we administered this vitamin to a number of patients with alopecia both total and regional, and to a number of patients with chronic bilateral skin eruptions of unknown etiology hitherto resistant to all forms of therapy. In the cases of alopecia we did not observe any regrowth of hair. In the cases with various skin diseases we have noted both negative and positive results. Several examples of rapid improvement in chronic eruptions following the administration of inositol are detailed. Our experience is still too recent to permit definite statements that inositol is or is not of benefit to any particular skin disease. However, it offers a new approach to the problems of dermatology and it is hoped that continued and extensive clinical trials will be made by the workers in this field.

There has been much comment as to the value of calcium pantothenate as an anti-gray hair factor. In our hands this has not been the case. Even though we used huge doses, from 100 to 500 mgms. per injection, we have only rarely noted any response in the hair. When response was apparent it occurred as a new growth of pigmented hair but no change in the existing gray hair was seen.

CONCLUSIONS

Riboflavin appears to be of value in the treatment of decubital ulceration. Following its administration the bed sores show rapid healing. The combined use of riboflavin and the entire B complex may be of even greater value in decubitus.

Inositol in doses of from one to two grams by mouth has no apparent harmful effect. In alopecia no beneficial results were noted. Rapid response in a variety of skin diseases following inositol administration were observed. It is hoped that the dermatologists will study this vitamin and clarify its field of usefulness.

Calcium pantothenate seems to have little value as a human anti-gray hair factor.

We wish to thank Dr. A. J. Beller, Dr. Milton Bodenheimer, Dr. Morris Dinnerstein, Dr. A. A. Epstein and Dr. S. Jahss for permission to use their cases.

REFERENCES

1. Axelrod, A. E. and Elvehjem, C. A.: *J. Biol. Chem.*, 140:725-735, 1941.
2. Emerson, G. A.: *Proc. Soc. Exp. Biol. and Med.*, 47:448-449, 1941.
3. Gavin, G. and McHenry, E. W.: *J. Biol. Chem.*, 139:485, 1941.
4. Henderson, L. M., McIntire, J. M., Waisman, H. A. and Elvehjem, C. A.: *J. Nutrit.*, 23:47-58, 1942.
5. Machella, T. E.: *J. Med. Sci.*, 203:114-119, 1942.
6. Martin, G. J., Thompson, M. R. and de Carvajal-Forero, J.: *Am. J. Dig. Dis.*, 8:290-295, 1941.
7. Sebrell, W. H. and Butler, R. E.: *Pub. Health Reports*, 53:2282-2284, 1938.
8. Unna, K., Richards, G. V. and Sampson, W. L.: *J. Nutrit.*, 22:553-563, 1941.
9. Williams, R. J., Weinstock, H. H., Jr., Rohrmann, E., Truesdail, J. A., Mitchell, H. K. and Meyer, C. E.: *J. Am. Chem. Soc.*, 61:454-457, 1939.
10. Williams, R. J. and Major, R. T.: *Science*, 91:246, 1940.
11. Woolley, D. W.: *Science*, 91:245-246, 1940.
12. Woolley, D. W.: *J. Biol. Chem.*, 136:113-118, 1940.
13. Woolley, D. W.: *Science*, 92:384-385, 1940.
14. Woolley, D. W.: *J. Biol. Chem.*, 139:29-34, 1941.
15. Woolley, D. W.: *Proc. Soc. Exp. Biol. and Med.*, 46:565-569, 1941.
16. Woolley, D. W.: *J. Biol. Chem.*, 142:963-964, 1942.
17. Woolley, D. W.: *J. Exp. Med.*, 75:277-284, 1942.
18. Woolley, D. W.: Personal communication.
19. Young, L.: *Biochem. J.*, 28:1435-1443, 1934.

DISCUSSION

DR. HARRY SHAY (Philadelphia): Mr. President, Ladies and Gentlemen: I think this contribution of Dr. Vorhaus extremely important. We are beginning to recognize the importance of some of the newer members of the B group, in animal and in human metabolism: We have had some experience with inositol in the human being, not in relation to skin changes, but were tempted to try inositol for its effect on fat metabolism based on the work of McHenry and his group at Toronto.

McHenry was able to produce cholesterol fatty changes in the liver in Vitamin B deficient animals given Biotin. He was able to prevent and cure these fatty livers with lipocaic and subsequently was able to do the same thing with inositol.

On the basis of these observations, we were prompted to study the effect of inositol in the diabetic, particularly in the diabetic with enlarged liver. Not knowing the required dose for the human being, we calculated our dosage on the basis of McHenry's results. He was able to produce beneficial results in his rats with a 5-milligram dose, and, considering the average rat as a half pound, we decided upon the use of 1.2 grams a day for the average patient.

We administered 1.2 grams per day to humans for a considerable period, without any ill effects, but, unfortunately, without being able to produce changes in the blood cholesterol levels or in the size of the liver.

The Metabolism of *meso*-Inositol in the Rat *

V. D. Wiebelhaus, Joseph J. Bethell and Henry A. Lardy

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

Received March 10, 1947

The almost ubiquitous occurrence of *meso*-inositol in plant and animal tissues, together with its structural relation to the 6-carbon sugars, (1) has prompted investigation and stimulated speculation upon its biological functions.

Inositol is required for the growth of certain yeasts (2, 3), a mutant strain of *neurospora* (4), and for animals under some conditions (5, 6). The vitamin like properties of inositol have been reviewed by Woolley (7).

Because of its chemical configuration inositol has long been considered a likely intermediate in the natural synthesis of aromatic compounds from carbohydrates. Fischer (8) has recently discussed the possibility of inositol being a "reserve" carbohydrate in living cells and suggested a possible role for inositol in the interconversion of various hexoses.

Since the first work of Vohl (9) in 1858, numerous investigations have been made of the metabolism of inositol by animals. Vohl, and later Needham (9), reported finding large amounts of inositol in the urine of human subjects. Needham also reported a vigorous excretion of inositol over long periods of time by polyuric rats on an inositol-free diet (10). These and other early studies of the metabolism of inositol by animals were hampered by lack of suitable quantitative methods for accurately determining this compound in biological material. No adequate proof has yet been offered that animal tissue can synthesize inositol, for Woolley (5) and Handler (11) have presented evidence that intestinal synthesis may furnish inositol to the rat. Furthermore, Woolley has shown (12) that the seeming synthesis of inositol by the chick embryo (Needham (13)) is actually a liberation of bound inositol from the yolk constituents.

The data on the absorption of inositol vary widely. Starkenstein (14) found only slight absorption by rabbits of injected or fed inositol. Mayer (15) reported that, when given orally to rabbits, 2.0-2.1% of the inositol appears in the urine but when given subcutaneously 24-52% of the dose is thus excreted. Anderson and Bosworth (16) found that when human subjects were fed inositol about 9% appeared in the urine and none was found in the feces. Working with dogs, Anderson (17) found inositol to be slowly absorbed and only a small portion of the material fed was excreted by the kidneys. He found no rise in the respiratory quotient.

The possible utilization of inositol as a carbohydrate has been investigated by many workers, mainly with inconclusive results. Das and Guha (18) reported increased oxygen consumption by brain, kidney and liver tissue of white rats when inositol was added. Young (19) could not confirm these results. Kulz (20) could find no increase in urinary sugar excretion of diabetic or phlorizinized animals after the administration of inositol. Von Mering (21) and Mayer (15) were unable to detect increased liver glycogen as a result of inositol administration.

Greenwald and Weiss (22) administered inositol orally to phlorizinized dogs over comparatively extended periods of time and found a slight but unmistakable increase in the urinary glucose:nitrogen ratio. They concluded that, in the dog, the inositol was slowly and incompletely converted to glucose.

Proof that at least some glucose is formed from inositol by the rat was presented by Stetten and Stetten (23). The injection of inositol containing stably bound deuterium into a phlorizinized rat resulted in the excretion of urinary glucose containing sufficient deuterium to indicate a minimum of 7% conversion of the injected inositol to glucose.

The experiments reported here were designed to determine the rate of absorption of inositol, its possible effect on liver glycogen, and the influence of inositol on starvation ketosis in the rat. Some determinations of the inositol content of certain rat tissues are also presented.

Experimental and Results

Quantitative determinations of inositol were made by the yeast (*S. carlsbergensis*) growth assay of Atkin *et al.* (24). Growth was measured by turbidity as determined with a photoelectric colorimeter using a filter with maximum transmission at 660 mμ. The assay range extended from 1 to 15γ per tube.

Absorption of Inositol

For the study of absorption the procedure of Cori (25) was followed. A series of white rats, all approximately 200 g. in weight, was fasted 24 hours. The animals were then given an aqueous solution of the test material by means of a small rubber catheter employed as a stomach tube. Dosages were proportional to $(\text{Weight})^{.73}$, a factor which Brody (26) has shown to parallel the "metabolically effective" weight of animals of all sizes more closely than do surface area formulae. For a 185 g. rat the dose amounted to 278 mg. of inositol. A calibrated hypodermic syringe was used to measure the solution; the test solution being followed by a minimum volume of distilled water to rinse the catheter. Controls were given equal volumes of distilled water by the same method and all animals were then placed in metabolism cages.

At various time intervals the animals were sacrificed. The upper and lower extremities of the gastrointestinal tract were ligated, the [illegible]

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the University Research Committee.

Arch. Biochem. 13:379-388, 1947

removed and quickly rinsed with distilled water. The contents of the tract were then washed into volumetric flasks with hot distilled water and, when cool, diluted to a definite volume. Urine specimens were collected from the time of dosage to the time of sacrifice. Aliquots of each sample were hydrolyzed by refluxing with 20% HCl for six hours to liberate all bound inositol for purposes of microbiological assay (27, 28). The hydrolyzates were evaporated to dryness under reduced pressure, taken up in water, adjusted to pH 4–5 and made up to a definite volume. Inositol was determined in both hydrolyzed and unhydrolyzed samples.

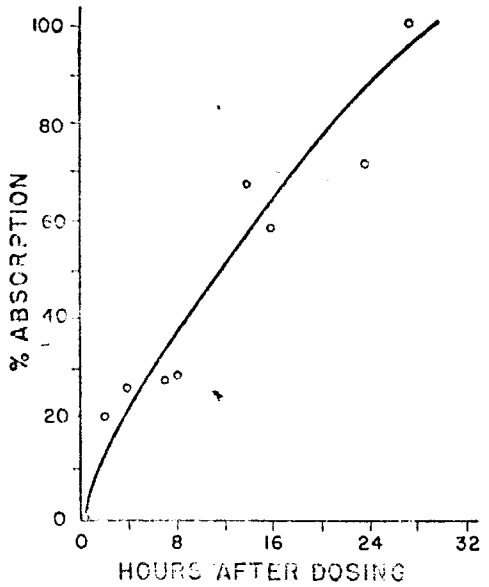


FIG. 1. Rate of inositol absorption in fasted rats.

The intestinal contents of several fasted rats were found to contain from 8 to 12 mg. of total inositol and this amount was therefore subtracted from all experimental values in calculating the amount of inositol which had been absorbed. The data obtained are summarized in Fig. 1. The rate of absorption was found to be comparatively slow, but appears to be complete at about 28 hours.

A comparison of the assay values for the hydrolyzed and unhydrolyzed samples indicated that no conversion of administered inositol to a bound form had occurred in the intestine. However, about one-half of the 8–12 mg. of inositol present in the tract of fasted animals (or of the experimental animals after absorption of the test dose was complete) was in a form not available to the strain of yeast used in these assays.

The urinary excretion of inositol varied from 3 to 5 mg./rat for a 24 hour period and was not significantly greater for the rats which had received from 260 to 335 mg. of inositol *per os* than for the fasted control rats. The limit of accuracy of the inositol assay permits the conclusion that urinary excretion accounts for less than 1% of the administered inositol.

The absorption of inositol monophosphate was also studied. From 150 to 200 mg. of the monophosphate isolated from soybean lipositol (27) was administered to each of eight 200 g. rats. The rate of absorption appeared to be more rapid than for inositol, especially during the first 2 hours. Approximately 80% of the dose had been absorbed in from 1 to 2 hours and complete absorption had occurred in rats killed at 12 and 24 hours. In all animals killed later than 2 hours after administering inositol monophosphate, almost all of the inositol in the intestinal contents was found to be in a form available to the assay organism. Since the original isolated inositol monophosphate gave a growth response equivalent to only 8–15% of its contained inositol, it appeared that the phosphoric ester was rapidly hydrolyzed in the intestine. The initial rapid disappearance of inositol might indicate an extremely rapid absorption of the inositol monophosphate *per se* with subsequent slow absorption of the free inositol which was presumably liberated by intestinal phosphatases.

Two of the rats which received inositol monophosphate excreted greater than normal amounts of inositol in their urine. The amounts were 10 mg. in 3 hours and 19 mg. in 8 hours, respectively. The other rats given inositol monophosphate excreted normal amounts of inositol and the differential assay showed no indication of bound forms of inositol in any of the urine specimens.

Tests for Glycogenesis

Previously reported attempts to demonstrate increased liver glycogen were either negative or inconclusive. However, in none of these studies, including the recent work of Sb(Ha...)[illegible] (23), were precautions taken to avoid glycogenolysis at death. Davenport and Davenport (29) and Cori (30) have shown that glycogen

breakdown is unavoidable unless the animal is first anesthetized and the tissue sample removed from the living animal. By employing this technique it seemed possible that any slight increment in liver glycogen resulting from inositol would be detectable.

White rats were fasted for 24 hours and then given aqueous solutions of either inositol or glucose by stomach tube. The dosages were again proportional to $W^{.75}$ and for a 175 g. rat amounted to 244 mg. of the compound fed at the lower level and 590 mg. at the higher level. Control rats were given equal volumes of distilled water. At various time intervals the rats were anesthetized by intraperitoneal injections of amytal. The livers were removed rapidly and immediately frozen in a bath of dry ice and acetone. They were then crushed and digested in hot 30% potassium hydroxide. The glycogen was separated and hydrolyzed by the method of Good *et al.* (31). Reducing sugar values were determined by the copper-iodometric method of Shaffer and Somogyi (32). The results are summarized in Table I.

TABLE I

Liver Glycogen in Inositol-Fed Rats

No. of rats[1]	Material fed	Dosage mg. $\times W^{.75}$	Hours from dosage to sacrifice	*Per cent* liver glycogen (based on wet weights of livers)
6	Water	—	4–6	.11±.05
10	Water	—	8–16	.08±.04
5	Water	—	19–28	.06±.03
3	Glucose	5.7	2–6	.51±.10
3	Glucose	5.7	12–16	.13±.05
1	Glucose	13.8	4	1.25
2	Glucose	13.8	8–12	.60±.20
2	Glucose	13.8	16–24	.09±.03
4	Inositol	5.7	4–8	.09±.03
5	Inositol	5.7	12–16	.11±.01
5	Inositol	5.7	19–28	.09±.02
2	Inositol	13.8	4–6	.07±.01
2	Inositol	13.8	8–16	.08±.02
3	Inositol	13.8	20–24	.07±.01
2	Inositol-monophosphate	5.7	2–4	.06±.03
1	Inositol-monophosphate	5.7	6	.16
1	Inositol-monophosphate	5.7	8	.08
1	Inositol-monophosphate	5.7	21	.13
1	Inosose	5.7	21	.07

[1] All animals were subjected to a preliminary fasting period of 24 hours.

Increased glycogen was found in the livers of the animals given glucose but no such increase over the control was shown by the animals given inositol at two levels of dosage. In the few animals given inositol monophosphate the liver glycogen appears to be sustained at a slightly higher level during the 8–21 hour period than in the case of the controls, but the difference is very slight.

Anti-Ketogenic Activity of Inositol

Since Stetten and Stetten (23) had found only a relatively minor conversion of administered inositol to glucose in their experiment and our absorption data indicated comparatively slow absorption of inositol it was concluded that a better measure (than glycogenesis) of the possible utilization of inositol as a carbohydrate would be its effect on ketosis.

A series of white rats (average weight about 180 g.) was fed the high fat-low protein diet of Tidwell and Treadwell (33) for 18 days. They were then fasted 48 hours and divided into four groups which received the following treatments respectively: I, 625 mg. of glucose by stomach tube; II, 625 mg. of inositol by the same route; III, 625 mg. of inositol injected intraperitoneally; IV, distilled water by stomach tube. The rats were kept in metabolism cages, given water *ad libitum* and the urines were collected during the following 24 hours. Total ketone bodies present in the urines were determined by the method of Tidwell and Treadwell (33).

The results are summarized in Table II. A marked antiketogenic effect was observed in the group which received glucose. Inositol given orally or intraperitoneally definitely alleviated the ketosis. The rats receiving glucose excreted less ketone bodies to the extent of 65 mg. less acetone than the controls, while those given inositol (Lots II and III) excreted an amount equivalent to 23 mg. less acetone. On this basis, inositol appeared to be approximately one-third as effective as glucose in alleviating ketosis in white rats.

As a check on the possibility that the differences in levels of the ketone bodies found in the urines might have been due to gluconeogenesis from body proteins, the individual urines were analyzed for total nitrogen by a semimicro Kjeldahl method as described by Umbreit and Bond (34). The data on urinary nitrogen, also given in Table II, show that the administration of either glucose or inositol

TABLE II

Effect of Inositol on Fasting Ketosis

Group	No. of rats	Material given	Ketone bodies determined as acetone in 24 hr. urine	Total nitrogen in 24 hr. urine
			mg.	*mg.*
I	4	625 mg. glucose orally in 2.4 ml. solution	15*	52*
II	4	625 mg. inositol orally in 2.4 ml. solution	56	58
III	4	625 mg. inositol—intraperitoneally in 2.4 ml. soln.	59	65
IV	4	2.4 ml. H_2O	80	74

* Each value represents the average daily excretion per rat in the respective group.

resulted in a decreased nitrogen excretion. The antiketogenic effects of these compounds were therefore not the result of gluconeogenesis from body proteins.

Distribution of Inositol in Rat Tissues

Since conversion of inositol to liver glycogen could not be detected, it was decided to study the possible deposition of inositol as such in various tissues of the rat.

Six white rats, averaging 250 g. each, were divided into three groups. Group I was taken directly from a stock ration and sacrificed. Groups II and III were fasted 24 hours. Group II was then given water by means of a catheter and Group III was given an equal volume of an inositol solution. The latter two groups were sacrificed six hours later. Blood samples and the livers, hearts and testes were removed. The tissues were digested and the inositol freed by refluxing for six hours in 20% HCl (28). The inositol in each tissue was determined by the microbiological assay. The results are summarized in Table III. The administration of inositol caused no appreciable changes in the tissue content of this compound, except in the case of heart. The hearts of the two fasted rats were found to contain 95 and 97 mg.-% of inositol while those of the rats which had been fed inositol contained 123 and 152 mg.-%, respectively. This marked increase is particularly interesting since Woodley (7) and Winter (35) had found comparatively high levels of inositol in beef heart.

TABLE III

Influence of Fasting and Inositol Feeding on the Inositol Content of Various Rat Tissues

Group No.	Fasting period	Material fed	Hours from dosing to sacrifice	mg.-% inositol*			
				Liver	Testes	Blood	Heart
	hrs.						
I	0	—	0	132	126	31	78
II	24	H_2O	6	155	115	34	97
III	24	800 mg. inositol	6	166	110	38	137

* Values given are the average for the 2 rats in each group.

Discussion

In spite of the precautions of removing the livers from amytalized animals and immediately freezing them, no significant increase in liver glycogen of fasted rats given inositol was obtained. Our experiments on absorption show that, at the level of dosage employed in the absorption and liver glycogen experiments, the inositol was completely absorbed only after 24–28 hours. Therefore, any glucose, or other similarly utilizable material, which may have been formed from the inositol may well have been metabolized in the fasted animals as rapidly as formed without causing any detectable deposition of liver glycogen. Although the utilization of inositol is evidently slow, it would appear that it is metabolized as carbohydrate by the rat, since its administration alleviates ketosis and at the same time reduces the amount of total nitrogen excreted in the urine. It is logical to suppose that an antiketogenic function may be observed if the material given is utilized as a carbohydrate in the fasted animal without a prior conversion to glycogen as such.

It is also of interest that there is a marked increase in the inositol content of the heart of the rat after inositol has been administered to the animal following a preliminary starving period of 24 hours.

Summary

1. When white rats of approximately 200 g. weight were given 250 mg. of inositol in aqueous solution, 24–28 hours were required for

complete absorption. Urinary excretion was found to account for less than 1% of the inositol given.

2. No increase in the liver glycogen of white rats was found after the administration of inositol.

3. Inositol, given orally or intraperitoneally, functioned antiketogenically in the white rat.

4. The fasting of rats for 24 hours did not influence the inositol content of their blood, liver, testis, or heart tissues. The oral administration of inositol to fasted rats resulted in an appreciable increase in the inositol content of the heart, but caused no appreciable change in the other tissues studied.

References

1. Fischer, H. O. L., Harvey Lecture XL, 156 (194[illegible]).
2. Eastcott, E. V., *J. Phys. Chem.* 32, 10[illegible] (1928).
3. Williams, R. J., [illegible], A., [illegible], H., and McMahan, J., *Univ. Texas Publ. No.* [illegible], 27 ([illegible]).
4. Beadle, G. W., *J. Biol. Chem.* [illegible], 68[illegible] (1944).
5. Woolley, D. W., *J. Exptl. Med.* 75, 277 (1942).
6. Cunha, T. J., [illegible], S., [illegible], P., and [illegible], G., *Proc. Soc. Exptl. Biol. Med.* [illegible], [illegible] (194[illegible]).
7. Woolley, D. W., *J. Nutrition* 28, [illegible] ([illegible]).
8. [illegible], M., [illegible] 17, 110 (185[illegible]).
9. [illegible], J., *Engl. Phys.* [illegible] 25, 1 (192[illegible]).
10. [illegible], J., *Biochem. J.* 18, [illegible] (1924).
11. [illegible], P., *J. Biol. Chem.* 1[illegible], 77 ([illegible]).
12. Woolley, D. W., *Proc. Soc. Exptl. Biol. Med.* 49, 540 (1942).
13. [illegible], J., *Biochem. J.* 18, [illegible] ([illegible]).
14. Starkenstein, E., *Z. exptl. Path. Therap.* 5, 378 (1908).
15. Mayer, P., *Biochem. Z.* 2, [illegible] (1907).
16. Anderson, R. J., and Bosworth, A. W., *J. Biol. Chem.* 25, 391 (1916).
17. Anderson, R. J., *J. Biol. Chem.* 25, 391 (1916).
18. [illegible], and [illegible], E. C., *Z. physiol. Chem.* 231, 157 (1935).
19. Young, L., *Proc. Soc. Exptl. Biol. Med.* 35, 507 (193[illegible]).
20. Külz, E., *Sitzber. Ges. Beförd. ges. Naturw. Marburg* (1875).
21. von Mering, J., *Arch. ges. Physiol.* 14, 274 (1877).
22. Greenwald, I., and Weiss, M. L., *J. Biol. Chem.* 31, 1 (1917).
23. [illegible], M. A., and [illegible], D., *J. Biol. Chem.* 164, 35 (1946).
24. [illegible], L., [illegible], A. S., [illegible], W. L., and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.* 15, 1[illegible] ([illegible]).
25. [illegible], C. F., *J. Biol. Chem.* [illegible], [illegible] ([illegible]).
26. [illegible], S., [illegible] New York, 1945.
27. Woolley, D. W., *J. Biol. Chem.* 1[illegible], [illegible] ([illegible]).

28. Woolley, D. W., *J. Biol. Chem.* 147, 581 (1943).
29. [illegible], A., and [illegible], H. K., *J. Biol. Chem.* 76, [illegible] (1928).
30. [illegible], G. T., *Am. J. Physiol.* 9[illegible], 557 (1936).
31. [illegible], C. A., [illegible], H., and Somogyi, M., *J. Biol. Chem.* 100, [illegible] (193[illegible]).
32. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.* 100, 695 (1933).
33. [illegible], H. C., and [illegible], C. R., *J. Biol. Chem.* 162, 155 (1946).
34. Umbreit, W. W., and Bond, V. S., *Ind. Eng. Chem., Anal. Ed.* 8, 276 (1936).
35. Winter, L. B., *J. Physiol.* 103, 27-P (1944).

The Journal of the American Medical Association

Published Under the Auspices of the Board of Trustees

VOL. 119, NO. 1 — COPYRIGHT, 1942, BY AMERICAN MEDICAL ASSOCIATION — CHICAGO, ILLINOIS — MAY 2, 1942

THE APPROXIMATE VITAMIN REQUIREMENTS OF HUMAN BEINGS

ROGER J. WILLIAMS, PH.D., D.SC.

Professor of Chemistry and Director, Biochemical Institute, University of Texas

AUSTIN, TEXAS

Most of the research on vitamins and nutrition is directed ultimately to the solution of problems of human nutrition. In a war situation such as ours it is particularly desirable that we be able to apply whatever we may know or learn to practical ends.

A fully adequate idea of the requirements of human beings for the various vitamins could presumably be obtained only as a result of a series of extended controlled studies using human subjects. Even if it were feasible to plan and carry out experiments of this type just as animal experiments are planned and carried out, individual differences, assuming that small numbers of subjects were used, would doubtless make the results very irregular.

Recently we have developed assay methods for a number of the B vitamins,[1] and because of an increased accumulation of information from various sources it becomes possible to state more definitely the vitamin contents of various foods and tissues. It appears now that this information makes it possible to obtain very simply a considerable amount of desired information regarding probable human requirements.

A study of the vitamin contents of (1) a well rounded mixed diet which might be recommended for human beings and (2) a widely used commercial animal food which promotes excellent performance in omnivorous animals shows that these more or less ideal diets, when compared on an isocaloric basis, contain a relatively constant amount of the various B vitamins. This comparison is made more striking when the carcass of a mammal (rat) is also compared. Again, on the same calory basis, the content of B vitamins agrees approximately and never is there disagreement by more than a factor of two. Where there is good agreement between these three independent materials there is a strong presumption that the amounts associated with 2,500 calories represent the approximate human requirement, assuming of course that the substance in question is actually a vitamin for human beings and cannot be synthesized in the body.

The indicated well rounded simplified diet, made up of natural foods, was formulated on the basis of modern nutritional knowledge but without regard to its content of B vitamins. Presumably any one of a number of other mixed diets would yield results similar in this regard. It is doubtful if any diet could be selected which a priori would be known to be superior to the one chosen. It contains "protective foods" but not in obvious excess and is sufficiently diversified so that every nutrient should be amply provided. Enough milk and orange juice were included to insure good supplies of calcium and ascorbic acid respectively, but other considerations regarding specific needs were disregarded. The diet as indicated was completely settled on before any calculations regarding its content of B vitamins were made.

No claim can be made that the assay values are without error or that the total values derived from their consideration are in any sense final. Individual samples of foodstuffs vary in their content of B vitamin, but since the diet is made up of a number of constitutents and often a number of samples were averaged, these variations doubtless tend to cancel out. There is no reason to think that the values obtained for any of the vitamins are consistently high. If enzymatic extraction is incomplete in any case, the values, of course, would tend to be low.

The animal food chosen (two samples obtained at different times of year) was a widely used commercial article suitable for rats, mice, dogs, cats and monkeys and which yields most excellent results, at least with the commoner laboratory animals. It is a mixed meat, milk and cereal diet containing various special additions including yeast and vitamin concentrates. Because the diet is the result of a large amount of research and yields excellent results, its general adequacy cannot be questioned. There are economic reasons why it would not be expected to contain the vitamins in excess. Concentrates and special additions are expensive and would naturally be used only on the basis of proved value.

An inspection of table 2 indicates that the following probably represent perfectly "safe" levels for daily intake of B vitamins by human beings, even under conditions of pregnancy and lactation: thiamine 3.2 mg., nicotinic acid 40 mg., riboflavin 3.7 mg., pantothenic acid 11 mg., biotin 0.14 mg., inositol 1,000 mg., pyridoxine 1.5 mg. and folic acid 1.0 mg. unit. It is not certain, of course, that all the B vitamins in the last four substances are actually required by human beings. Regardless of this fact, the vitamins actually occur in foods to about the extent indicated.

The National Research Council Committee on Foods and Nutrition has recently given careful study to the vitamin requirements of human beings. On the basis of available information the thiamine requirement, on a 2,500 calory basis, has been estimated at 1.5 mg. daily. During pregnancy and lactation this is raised

I have received valuable assistance from my colleagues. The Williams-Waterman Fund of Research Corporation made a special grant and the Clayton Foundation of Houston, Texas, furnished the support which has made our testing program possible.

1. Studies on the Vitamin Content of Tissues: I. University of Texas Publication 4137, 1941.

(figured on the same calory basis) to 1.8 mg. and 1.9 mg. respectively. The lowest figure is about the content of cow's milk and is higher than the content of human milk and therefore may be high enough. However, on the basis of the figures given in table 2, one might conclude that the values given are too low for absolute safety. Considering the whole rat carcass as a "complete" food, its thiamine content appears low in comparison until the low carbohydrate content is considered.

The National Research Council Committee has estimated the nicotinic acid requirement of man as 15 to 20 mg. a day (2,500 calories). Elvehjem has placed it at 25 mg. a day. The two diets furnish an adequate supply even on the latter basis. The higher content of the rat carcass may be correlated with the rat's ability to synthesize nicotinic acid.

The human riboflavin requirement has been estimated at 2.2 to 2.5 mg. daily (2,500 calories). According to the figures given in table 2, one would conclude that

TABLE 1.—*Vitamin B Content of Foods*

Constituent	Per Cent of the Calories	Thiamine, Mg.	Nicotinic Acid, Mg.	Riboflavin, Mg.	Pantothenic Acid, Mg.	Biotin, Mg.	Inositol, Mg.	Pyridoxine, Mg.	Folic Acid, Mg. Unit
Whole milk.....	20	0.3	0.4	1.4	2.2	0.06	100	0.20	0.100
Whole wheat.....	30	1.0	11.5	0.31	2.4	0.01	350	0.41	0.366
Lean meats									
Beef.....	9	0.07	10.0	0.4	1.0	0.01	21	0.11	0.043
Pork.....	9	1.5	7.0	0.2	0.7	0.01	8	0.11	0.025
Liver.....	2	0.07	5.0	1.0	2.4	0.13	23	0.02	0.057
Potatoes.....	10	0.2	3.6	...	1.0	0.002	87	0.06	0.390
Butter.....	5	*	*	*	*	*	*	*	*
Eggs.....	6	0.2	*	0.04	0.2	0.002	1	*	*
Fresh peas.....	4	0.15	2.0	0.11	0.7	0.014	162	0.05	0.124
Fresh carrots.....	4	0.1	0.6	0.15	0.6	0.012	235	0.16	0.285
Orange juice.....	2	*	*	*	*	*	*	*	*
Total.....	100	3.6	40.1	3.67	11.2	0.25	987	1.77	1.39

* The amount of these foods is small enough so that they contribute a negligible amount of the B vitamin in question to the diet.

for perfect safety this figure should be increased by about 60 per cent.

No one has, so far as I know, ventured an estimate of the human requirement of pantothenic acid, but it appears safe now to conclude that it cannot be far from 10 mg. a day. The validity of this estimate is increased by the fact that cow's milk yielded 10.5 mg. per 2,500 calories, and human milk 10.9 mg. per 2,500 calories.

The pyridoxine requirement, following the same line of reasoning, appears to be about 1.5 mg. a day, that of "folic acid" (assuming it to be required) about 1 mg. unit a day, and that of biotin about 0.15 mg. a day.

Inositol is predominantly from vegetable sources, as shown in table 1, and the amount associated with 2,500 calories of food varies tremendously, depending on the source. A good mixed diet yields 1 Gm. a day or more.

Assays of various organisms from different portions of the biologic kingdom (mammals, insects, bacteria, yeasts, higher plants) indicate that all eight of the B vitamins considered are always present. There appears, particularly in the case of thiamine, nicotinic acid, riboflavin and pantothenic acid, a definite tendency toward parallelism in the content of whole organisms. Yeasts, for example, are five to ten times as rich as the mixed diet in each of these four vitamins. Insects (cockroaches and termites), on the other hand, are in every case two to four times as rich in these vitamins as the mixed diet. The other B vitamins tend to be more evenly distributed in the lower and higher forms of life. It thus appears that the tentative human requirements mentioned are parallel to but much lower than the amounts (figured on an isocaloric basis) which insect eating birds or yeast eating insects obtain.

TABLE 2.—*Vitamin B Content of Various Materials (2,500 Calories)*

	Thiamine, Mg.	Nicotinic Acid, Mg.	Riboflavin, Mg.	Pantothenic Acid, Mg.	Biotin, Mg.	Inositol, Mg.	Pyridoxine, Mg.	Folic Acid, Mg. Unit
Mixed diet.....	3.6	40.1	3.67	11.2	0.25	987	1.77	1.39
Dog food.....	2.8-4.4	40-24	3.7-6.2	10-10.9	0.114-0.14	1,170-2,900	1.86-1.13	0.66-0.91
Rat carcass.....	1.86	68.0	4.03	14.9	0.124	217	0.93	1.56

It is obvious that for various reasons (economic inability, use of decorticated grains, losses in cooking and cooking water, use of refined sugar and other "refined" foods) most people do not get as much of the various B vitamins as they probably should have.

What can be done to supplement this lack? One of the means which has been indorsed by some members of the medical profession is the prescription of vitamin B complex preparations, which are usually derived from yeast or liver and often fortified with those synthetic vitamins which are available.

Unfortunately, while yeast and liver are rich sources of B vitamins, both known and unknown, they do not necessarily yield extracts which are extraordinarily rich. The B vitamins occur in bound form and are freed only by some type of digestion (autolysis or otherwise). When digestion is used to free the vitamins, much other soluble material is formed which contaminates the extract. One commercial brewers' yeast extract (from autolyzed yeast) was assayed for the eight B vitamins considered and found to be a good source of each. A calculation showed however that, in order to get enough of the B vitamins to equal the intake on a good diet, one would have to consume

TABLE 3.—*Constituents of Average Daily Adult Dose of "Vitamin B Complex" as Sold by Six Representative Drug Houses*

	Thiamine, Mg.	Riboflavin, Mg.	Nicotinic Acid, Mg.	Pantothenic Acid, Mg.	Pyridoxine, Mg.
1.....	2.5	0.62	5.0	0.0	0.330
2.....	1.0	1.0	0.15	0.225	0.025
3.....	3.0	2.0	20.0	1.0	1.0
4.....	0.75	0.25	...	...	0.25
5.....	2.5	0.7	35.0	...	0.56
6.....	3.0	1.0	10.0	0.6	0.5
Amounts contained in well rounded diet (2,500 calories).....	3.0	3.7	40.0	11.0	1.5

10 per cent of one's calories in the form of this yeast extract (7 to 8 teaspoons of dry powder). In a preparation such as this the physician could hardly expect to find a therapeutic agent of pronounced value. Extracts or preparations which are worthy of consideration should be plainly labeled as to their content of the various B vitamins and enough should be present to allow significant dosage. For correction of a deficiency

it may be desirable in some cases to give several times the ordinary daily intake.

A survey of a number of representative vitamin B complex preparations with regard to their content of B vitamins is summarized in table 3. It is apparent that these preparations are in general wholly inadequate as sources of the vitamin B complex. Several of them may be of value because of their fortification with thiamin, nicotinic acid or riboflavin, but they fail to contain anything like "balanced" amounts of the various B vitamins.

My purpose in this article is to point out that the approximate human requirements for various of the B vitamins can now be estimated with some reliability. Many American diets are doubtless low in these essential substances. Attempts to correct these deficiencies must be made intelligently and with full knowledge of what the requirements are and the contents of the proposed remedial agents. The present economic waste involved in the production and sale of preparations of questionable value must be large.